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**Canine Idiopathic Pulmonary Fibrosis in West  
Highland White Terriers: Natural History and  
TGF- $\beta$  Related Molecular Pathways**



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# CANINE IDIOPATHIC PULMONARY FIBROSIS IN WEST HIGHLAND WHITE TERRIERS: NATURAL HISTORY AND TGF- $\beta$ RELATED MOLECULAR PATHWAYS

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ACADEMIC DISSERTATION

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# ABSTRACT

Canine idiopathic pulmonary fibrosis (CIPF) is an incurable fibrosing lung disease occurring mainly in West Highland White Terriers (WHWTs). The clinical picture of CIPF has many similarities with human idiopathic pulmonary fibrosis (IPF). Signs include dry cough, exercise intolerance, and respiratory difficulties. Prognosis for CIPF and human IPF is poor, and only limited treatment options are available. Histopathological CIPF shares features of both human usual interstitial pneumonia (UIP), the pathological counterpart of human IPF, and other human idiopathic interstitial pneumonia, the non-specific interstitial pneumonia (NSIP). Chronic bronchitis (CB) is the main differential diagnosis for CIPF, but antemortem differentiation can be challenging, as surgical lung biopsies are seldom taken and no clinically useful biomarkers are currently available. The natural history of CIPF has not been previously studied and little is known about the molecular pathophysiology of CIPF.

This thesis describes the clinical course, survival, and evaluation of exercise intolerance using the 6-minute walk test in CIPF WHWTs. Bronchoalveolar lavage fluid (BALF) protein expression was studied to find potential biomarkers for CIPF, and aspects of transforming growth factor (TGF)- $\beta$  related molecular pathways in pathogenesis of CIPF were investigated. In addition, results of TGF- $\beta$  signaling activity and its known extracellular matrix (ECM) regulatory proteins, latent TGF- $\beta$  binding protein (LTBP)-1 and fibrillin-2, in CIPF were compared with findings in human IPF/UIP and NSIP.

The follow-up study showed that CIPF has a significant negative impact on life expectancy of diseased dogs. The median CIPF-specific survival after onset of clinical signs in WHWTs was 2.7 years, but individual survival varied considerably from only a few months to over 4 years. This variance indicates that CIPF may have either a slow or a rapid disease progression, as also seen in human IPF. In addition, during the disease course acute exacerbations (AEs) occurred. The 6-minute walk distance proved to be an easy and noninvasive parameter to evaluate lung function and level of exercise intolerance in CIPF WHWTs. No significant prognostic factors were identified.

The quantitative comparison of BALF proteomes obtained from CIPF WHWTs, CB dogs, and healthy dogs revealed similar changes for CIPF and CB, which suggests a common response to disease processes in these otherwise different lung diseases. Specific biomarkers for CIPF were not identified.

The immunohistochemical stainings suggested that increased TGF- $\beta$  signaling and its ECM regulatory proteins LTBP-1 and fibrillin-2 are part of the molecular pathophysiology of CIPF, as also seen in human IPF/UIP and

NSIP. We demonstrated strong expression of activin B, a member of the TGF- $\beta$  superfamily, in the altered alveolar epithelium of WHWTs with CIPF. Furthermore, activin B was detected in BALF of CIPF WHWTs, most notably in samples from dogs with AE, but not in BALF of healthy WHWTs. This novel finding suggests that activin B is part of the CIPF pathophysiology and might act as a potential marker of alveolar epithelial damage.

Our findings add important new knowledge about the natural history and molecular pathophysiology of CIPF in WHWTs and show similarities between CIPF and human IPF. Better understanding of the complex pathogenesis of CIPF and human IPF is crucial for developing novel diagnostic tools and treatment strategies for these yet incurable diseases.

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Lilja-Maula L, Laurila HP, Syrjä P, Lappalainen AK, Krafft E, Clercx C, and Rajamäki MM (2014) Long-term outcome and use of 6-minute walk test in West Highland White Terriers with idiopathic pulmonary fibrosis. *Journal of Veterinary Internal Medicine*, 28:379-385.
- II Lilja-Maula L, Palviainen M, Heikkilä HP, Raekallio MR, and Rajamäki MM (2013) Proteomic analysis of bronchoalveolar lavage fluid samples obtained from West Highland White Terriers with idiopathic pulmonary fibrosis, dogs with chronic bronchitis, and healthy dogs. *American Journal of Veterinary Research*, 74:148-154.
- III Lilja-Maula L, Syrjä P, Laurila HP, Sutinen E, Rönty M, Koli K, Rajamäki MM, and Myllärniemi M (2014) Comparative study of transforming growth factor- $\beta$  signalling and regulatory molecules in human and canine idiopathic pulmonary fibrosis. *Journal of Comparative Pathology*, 150:399-407.
- IV Lilja-Maula L, Syrjä P, Laurila HP, Sutinen E, Palviainen M, Ritvos O, Koli K, Rajamäki MM, and Myllärniemi M. Upregulation of alveolar levels of activin B, but not activin A, in lungs of West Highland White Terriers with canine idiopathic pulmonary fibrosis and diffuse alveolar damage. Accepted to be published in *Journal of Comparative Pathology*, November 2014.

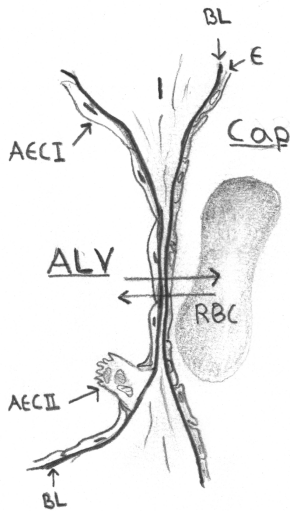
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# ABBREVIATIONS

AE	acute exacerbation
AEC	alveolar epithelial cell
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CB	chronic bronchitis
CIPF	canine idiopathic pulmonary fibrosis
DAD	diffuse alveolar damage
2D-DIGE	two-dimensional differential gel electrophoresis
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ET-1	endothelin-1
HRCT	high-resolution computed tomography
IPF	idiopathic pulmonary fibrosis
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LTBP	latent transforming growth factor- $\beta$ binding protein
6MWD	six-minute walk distance
6MWT	six-minute walk test
NSIP	non-specific interstitial pneumonia
PIIINP	procollagen type III amino terminal peptide
TGF- $\beta$	transforming growth factor- $\beta$
TOFMS	time-of-flight mass spectrometer
UIP	usual interstitial pneumonia
WHWT	West Highland White Terrier

# 1 INTRODUCTION

The primary function of the canine respiratory system, consisting of upper and lower respiratory components, is gas exchange in which oxygen and carbon dioxide are transported between the environment and the tissues. Other functions of the respiratory system include thermoregulation, metabolism of endogenous and exogenous substances, and protection against inhaled particles and infectious agents. The canine lower respiratory tract comprises the trachea, the main bronchi, and the right and left lung. Ventilation passes air through the trachea and bronchial tree into the terminal air spaces of the respiratory system, the alveoli, where the gas exchange between the air and blood occurs. The air-blood barrier consists of a thin layer of surfactant lining the alveolar surface, the alveolar epithelial layer formed by flattened squamous type I cells (alveolar epithelial cell, AECI) and cuboidal type II cells (AECII), alveolar basal lamina, variable thickness interstitium, and capillary endothelium (Figure 1). The diffusion of oxygen and carbon dioxide occurs through a thin portion of the alveolar-capillary complex, where the type I cell is separated from the capillary endothelial cell by a single basal lamina shared by the two cell types (Ross et al. 1995, Robinson 1997).



**Figure 1** Illustration of oxygen (arrow to right) and carbon dioxide (arrow to left) diffusion between the alveolus (ALV) and red blood cell (RBC) within the pulmonary capillary (Cap). AECI/II, alveolar epithelial cell type I/II; BL, basal lamina; E, capillary endothelium; I, interstitium.

Normal tissue repair is essential to allow tissues and organs to recover after an insult caused by a wound or a disease. The acutely or moderately injured tissue or organ should in the normal healing process be almost completely restored. However, if the injury persists, an impaired remodeling process with excess extracellular matrix (ECM) deposition, mainly collagen, occurs, causing fibrosis and organ dysfunction (Micallef et al. 2012). Altered ECM and fibrotic diseases of various organs, including the heart, kidney, liver, and lung, have been reported in dogs (Ide et al. 2001, Spee et al. 2006, Aupperle et al. 2009, Syrjä et al. 2013) as well as in humans (Blobe et al. 2000).

Canine idiopathic pulmonary fibrosis (CIPF) is a breed-predisposed disease of unknown etiology, occurring particularly in West Highland White Terriers (WHWTs) (Norris et al. 2005). In humans, idiopathic pulmonary fibrosis (IPF) is considered to be a consequence of repeated alveolar epithelial injury of unknown cause, leading to an abnormal repair process and accumulation of collagen in the pulmonary interstitium (King et al. 2011). The thickened interstitium hampers gas exchange and eventually leads to respiratory failure in both human IPF (Katzenstein et al. 2008) and CIPF (Norris et al. 2005). Clinical and histopathological features of CIPF in WHWTs and their resemblance to features of human IPF have been reported earlier (Heikkilä et al. 2011a, Syrjä et al. 2013). However, the natural course of CIPF has not been previously described, and little is known about its pathogenesis. In addition, noninvasive diagnostic tools, such as biomarkers from blood or bronchoalveolar lavage fluid (BALF), would be highly beneficial for CIPF diagnostics since high-resolution computed tomography (HRCT) or lung biopsies are not widely available in clinical veterinary practice. Revealing the complex pathogenetic mechanisms of CIPF and human IPF is critical for development of novel treatment strategies, as currently no curative treatment for these fatal diseases exists and the causative agents are unknown (Heikkilä et al. 2011a, Raghu et al. 2011).

The purpose of this thesis was to describe the natural course of CIPF in WHWTs, to uncover potential biomarkers, and to elucidate the molecular pathogenesis of CIPF.

## **2 REVIEW OF THE LITERATURE**

### **2.1 IDIOPATHIC PULMONARY FIBROSIS (IPF)**

#### **2.1.1 DEFINITION, HISTOPATHOLOGICAL FEATURES, AND PATHOGENESIS**

IPF, both in dogs (Norris et al. 2005, Heikkilä et al. 2011a) and in humans (Raghu et al. 2011), is a progressive, fibrosing lung disease of unknown cause affecting primarily the pulmonary interstitium. In dogs, it appears to be breed-specific, occurring mainly in terriers, most notably in WHWTs (Corcoran et al. 1999, Lobetti et al. 2001, Heikkilä et al. 2011a). In man, most cases of IPF are considered to be sporadic and the familial forms account for less than 5% (Raghu et al. 2011). An IPF-like condition has also been reported in cats (Cohn et al. 2004, Williams et al. 2004), and a distinct form of pulmonary fibrosis, equine multinodular pulmonary fibrosis associated with equine herpesvirus 5, has been described in horses (Williams et al. 2007, 2013).

The main histopathological finding in WHWTs with CIPF is mature diffuse interstitial fibrosis of the alveolar walls (Syrjä et al. 2013). The histopathological pattern of IPF in humans is usual interstitial pneumonia (UIP). The IPF/UIP belongs to the group of idiopathic interstitial pneumonias, and its differentiation from the very similar subtype of idiopathic interstitial pneumonia, the non-specific interstitial pneumonia (NSIP), and especially from its fibrosing form, can be difficult (Katzenstein et al. 2008, Meltzer and Nobel 2008). The cellular form of NSIP is characterized by a chronic inflammatory cell infiltrate with minimal fibrosis, and its differentiation from UIP is usually straightforward (Katzenstein et al. 2008). CIPF in WHWTs shares histopathological features of both UIP and fibrotic NSIP (Syrjä et al. 2013). For clinical and histopathological terminology, see Table 1. The diffuse interstitial fibrosis and variable grade of interstitial inflammation seen in CIPF resemble fibrosing NSIP histopathologically, but the alveolar architectural distortion (i.e. honeycomb change), seen in areas of more severe fibrosis in CIPF is typical of UIP (Katzenstein et al. 2008, Syrjä et al. 2013). Fibroblastic foci, which are composed of fibroblasts and myofibroblasts and indicate an active, ongoing disease process, are characteristic of human UIP (Katzenstein et al. 2008), but are not seen in CIPF (Syrjä et al. 2013).

**Table 1** *Clinical and histopathological terminology.*

<b>Clinical diagnosis</b>	<b>Histopathological picture</b>
IPF	UIP
NSIP	fibrosing NSIP or cellular NSIP
CIPF	CIPF

IPF, idiopathic pulmonary fibrosis; UIP, usual interstitial pneumonia; NSIP, non-specific interstitial pneumonia; CIPF, canine idiopathic pulmonary fibrosis.

The pathogenesis of human IPF is still incompletely understood, but evolving evidence indicates that the pathogenesis is epithelial-driven (King et al. 2011). Repeated injuries to alveolar epithelial cells with subsequent destruction of the alveolar basement membrane lead to aberrant epithelial repair (Coward et al. 2010). While many factors have been proposed to contribute to the epithelial injury, such as environmental factors, chronic viral infections, and chronic silent gastroesophageal microaspiration, the etiology of human IPF remains unknown (King et al. 2011). In reaction to the injurious agent, the AECI cells, which normally constitute more than 90% of alveolar wall area and are responsible for gas exchange, undergo apoptosis (Prasse and Müller-Quernheim 2009). As AECI cells are unable to replicate, accumulation of hyperplastic AECII cells occurs as a regenerative attempt. The repetitively activated AECII cells produce a variety of growth factors and chemokines, most notably transforming growth factor (TGF)- $\beta$ 1, leading to excess ECM formation and development of IPF (Coward et al. 2010). Autoimmune disorders have been suggested to have role in the pathogenesis of idiopathic NSIP (Romagnoli et al. 2011).

As marked atypia of AECII has been shown to be a consistent finding in lungs of CIPF WHWTs, it is likely that AECs and possibly exhaustion of their reparative capacity are important in the pathogenesis of CIPF (Syrjä et al. 2013). Microarray analyses of gene expression profiles in lungs of dogs with CIPF have revealed several potential genes that take part in the pathogenesis of CIPF (Krafft et al. 2013). The biological functions associated with these genes are related to cellular growth and proliferation, inflammation and immune response, and developmental processes (Krafft et al. 2013). In addition, activation of TGF- $\beta$ 1 pathways (Krafft et al. 2012) and upregulation of collagenolytic matrix metalloproteinase-9 (Heikkilä et al. 2011b) have been suggested to take part in the pathogenesis of CIPF. The higher serum TGF- $\beta$ 1 concentrations observed in healthy WHWTs relative to other breeds might partly explain the breed susceptibility for CIPF (Krafft 2011a, Krafft et al. in press).

## **2.1.2 CLINICAL FEATURES AND DIAGNOSIS**

CIPF affects mainly middle-aged to older WHWTs and causes gradually worsening respiratory signs, including dry cough and exercise intolerance (Corcoran et al. 1999, Heikkilä et al. 2011a). In addition, pulmonary hypertension is a relatively common complication seen in WHWTs with CIPF (Schober and Baade 2006). Clinical diagnosis of CIPF can be challenging, as clinical signs are nonspecific and slowly developing. The condition can be confused with other respiratory diseases, especially chronic bronchitis (CB), and with cardiac diseases (Corcoran et al. 1999). Tentative diagnosis is done based on signalement and clinical findings featuring velcro crackles on auscultation, low arterial partial pressure of oxygen, increased alveolar-arterial oxygen gradient, and a moderate to severe bronchointerstitial pattern in thoracic radiography. Additional diagnostics includes bronchoscopy and bronchoalveolar lavage (BAL) for eliminating differential diagnoses and HRCT to further confirm the CIPF diagnosis in the absence of surgical lung biopsy (Corcoran et al. 1999, Johnson et al. 2005, Heikkilä et al. 2011a). As antemortem surgical biopsies are seldom taken from dogs, histopathological confirmation of the diagnosis is usually performed after death (Johnson et al. 2005).

Human IPF patients experience exertional dyspnea, chronic dry cough, finger clubbing, and bibasilar inspiratory crackles on auscultation (Raghu et al. 2011). The disease occurs with older age, the mean age at presentation being 66 years. As in dogs, the slow onset of symptoms can be attributed to aging or other cardiac or respiratory diseases, delaying the diagnosis for several months to years (Meltzer and Noble 2008). In humans, diagnosis of IPF requires the presence of a UIP pattern on HRCT in patients not subjected to surgical lung biopsy (Raghu et al. 2011). The natural history of human IPF varies. Most patients have a gradual decline in functional status after diagnosis, but some may have a rapidly progressive disease course or a step-like progression, in which periods of relative stability are interrupted by acute exacerbations (AEs) (Kim et al. 2006). AEs of IPF are defined as combination of sudden worsening of dyspnea within 30 days with new lung infiltrates in HRCT and no evidence of pulmonary infection or other underlying cause, such as pulmonary embolism or left heart failure (Collard et al. 2007). AEs are associated with high mortality and a histopathological pattern of diffuse alveolar damage (DAD) (Rice et al. 2003, Kim et al. 2006, Ley et al. 2011). DAD has also been demonstrated in WHWTs with CIPF, along with consequent organizing luminal fibrosis, suggesting that more rapid progression of the fibrosis through organizing DAD can occur in dogs as well (Syrjä et al. 2013).

### **2.1.3 SIX-MINUTE WALK TEST (6MWT) FOR EVALUATION OF EXERCISE TOLERANCE**

The six-minute walk test (6MWT) is a submaximal exercise test commonly used to measure functional exercise capacity before and after medical intervention in human patients with a moderate or severe heart or lung disease. It can also be used to measure one-time functional status and even predict the mortality from heart or lung disease (ATS 2002, Enright 2003). In dogs, the use of 6MWT has been assessed in a limited number of studies with healthy dogs (Ferreira et al. 2010, Gault et al. 2010, Swimmer and Rozanski 2011), dogs with induced congestive heart failure (Boddy et al. 2004), and dogs with various pulmonary diseases (Swimmer and Rozanski 2011). The 6MWT has been found to be an easy and reproducible test for screening exercise tolerance.

The test is considered safe and reflects well the activities of daily living (ATS 2002, Enright 2003). The primary outcome is the distance walked (6MWD). In addition, other measurements, such as oxygen desaturation using pulse oximetry and in humans level of fatigue or dyspnea, can be obtained before, during, and after 6MWT (ATS 2002, Enright 2003). The 6MWT is widely used in clinical practice to evaluate human IPF patients and has been shown to be a reliable, valid, and responsive measure of exercise tolerance in IPF patients (du Bois et al. 2011). Desaturation during the test and the distance walked have been linked to increased risk of death in IPF patients (Lama et al. 2003, Caminati et al. 2009). In addition, in human IPF patients, a decline of 24-45 m over 6 months represents a clinically important difference in the risk of death at 1 year, and this change could be used as a guideline for change in therapy or as an endpoint in clinical trials (du Bois et al. 2011). In dogs, the distance walked has been shown to decrease after an induced heart failure (Boddy et al. 2004), and dogs with respiratory disease walked shorter distances than healthy dogs (Swimmer and Rozanski 2011). However, in the latter study, the dogs suffering from different respiratory diseases were older and of smaller breed than controls (Swimmer and Rozanski 2011). Age- and breed-matched controls are important when comparing 6MWD results in dogs, as leg length and age are associated with walking distance (Enright 2003, Swimmer and Rozanski 2011). No studies reporting the use of 6MWT as a prognostic factor or as a measurement of treatment outcomes in dogs with lung or heart disease have been published.

### **2.1.4 SURVIVAL AND PROGNOSTIC FACTORS**

The median survival of WHWTs with CIPF has been reported in a retrospective study to be 16 months from the onset of clinical signs and 7 months from diagnosis, but CIPF is speculated not to shorten the life expectancy since the disease affects middle-aged to older WHWTs (Corcoran et al. 1999). In humans, median survival of IPF patients from diagnosis is only 2-3 years, however, this might be underestimate in some patient groups



(Raghu et al. 2011). Fibrotic NSIP has a better prognosis, median survival being 6-13.5 years (Kim et al. 2006), and the cellular form has an excellent long-term outcome (Park et al. 2009).

No prognostic factors affecting the survival of WHWTs with CIPF have been published. In human IPF, the search for prognostic factors has been extensive since the course of the disease can vary greatly among patients, from rapidly to slowly progressive to a step-like process (Kim et al. 2006). Several factors have been associated with poor survival in humans, including decreased walking distance in 6MWT (Caminati et al. 2009), increasing grade of interstitial fibrosis on thoracic radiographs (Schwartz et al. 1994), fibrosis score, and traction bronchiectasis in thin-section computed tomography (Sumikawa et al. 2008), increased BAL neutrophilia (Kinder et al. 2008), and the presence of pulmonary hypertension (Andersen et al. 2012).

### **2.1.5 TREATMENT OPTIONS**

Very limited information is available regarding treatment options for CIPF. Medications commonly used to treat CIPF include corticosteroids, bronchodilators, and antimicrobials (Corcoran et al. 1999). It is currently unknown whether these medications have an effect on the clinical course of CIPF, but lung fibrosis in humans is unresponsive to these commonly used medications (Raghu et al. 2011). However, many dogs with CIPF have concurrent bronchial changes (Heikkilä et al. 2011a, Syrjä et al. 2013) and some seem to respond to corticosteroid treatment (Corcoran et al. 1999). In addition, the majority of patients with NSIP are treated with corticosteroids and cytotoxic agents and seem to benefit from these (Park et al. 2009). If pulmonary hypertension is present, sildenafil, which has been evaluated in the treatment of pulmonary hypertension in dogs (Brown et al. 2010), can be tried.

The benefits of the first and as yet only antifibrotic drug, pirfenidone, in human patients with IPF have been demonstrated in many clinical trials in recent years (Azuma et al. 2005, Noble et al. 2011, King et al. 2014). Pirfenidone reduces the decline in lung function and prolongs progression-free survival (Noble et al. 2011, King et al. 2014). The exact receptor for pirfenidone is unknown, but it has combined anti-inflammatory, antioxidant, and antifibrotic effects in experimental models of fibrosis, as it regulates the activity of TGF- $\beta$  and tumor necrosis factor- $\alpha$ , and inhibits fibroblast proliferation and collagen synthesis (Iyer et al. 1999, Di Sario et al. 2002, Nakazato et al. 2002). Although the pharmacokinetics of pirfenidone in dogs has been evaluated (Bruss et al. 2004), no reports on its clinical use in dogs exist, and the high price of the drug will (likely) hinder treatment trials. In addition to pirfenidone, one of the most important recent discoveries in the therapeutic field of human IPF has been that the previously used standard triple therapy (prednisone, azathioprine, and N-acetylcysteine) was

demonstrated to be harmful, possibly due to the toxic actions of the immunosuppressive agents, and is no longer used to treat IPF (Raghu et al. 2012, Martinez et al. 2014). Nintedanib, a triple tyrosine kinase inhibitor, has recently been shown to slow disease progression in human IPF patients (Richeldi et al. 2014). Lung transplantation is an appropriate option for only some IPF patients (Raghu et al. 2011). Despite recent therapeutic advances, IPF is a fatal disease and improving the outcomes for IPF patients remains a challenge (Raghu and Thickett 2013).

### **2.1.6 SEARCH FOR POTENTIAL BIOMARKERS**

According to the Biomarkers Definition Working Group (2001), a biological marker (biomarker) can be defined as follows: “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. However, the term nowadays usually refers to molecular or biochemical markers (Frangogiannis 2012). An optimal biomarker should be noninvasive and easy to obtain, e.g. by blood sampling or urine collection, and it should be able to be analyzed using standard measurement techniques (Veenstra et al. 2005, Prasse and Müller-Quernheim 2009). The epithelial lining fluid collected during BAL reflects the airway protein composition and makes it a suitable material for a biomarker search in lung diseases (Lenz et al. 1990, Noël-Georis et al. 2001, Wattiez and Falmagne 2005, Magi et al. 2006). Modified protein expression in human BALF for various respiratory diseases, including IPF, has been reported (Noël-Georis 2001, Magi et al. 2002, Wattiez and Falmagne 2005), but no BALF proteomic studies regarding canine lung disease have been previously published.

The search for disease-specific biomarkers in BALF or serum of humans with IPF has been extensive in recent years (Kinnula et al. 2009, Prasse and Müller-Quernheim 2009). A biomarker that could distinguish between UIP and NSIP, reflect disease activity, or predict who will have IPF in the future would be most beneficial (Prasse and Müller-Quernheim 2009, Vij and Noth 2012). Unfortunately, although numerous biomarkers have been proposed for human IPF, none have yet been validated or implemented in clinical practice (Kinnula et al. 2009, Prasse and Müller-Quernheim 2009, Vij and Noth 2012). In dogs, as HRCT is not widely available in clinical veterinary practice for confirming CIPF diagnosis, and surgical biopsies are seldom taken, noninvasive biomarkers from blood or BALF are even more needed. Currently, for CIPF, serum and BALF endothelin-1 (ET-1) concentrations (Krafft et al. 2011b), BALF procollagen type III amino terminal peptide (PIIINP) concentrations (Heikkilä et al. 2013), and serum chemokine (C–C) ligand 2 (CCL2) (Krafft et al. 2013) have been proposed as potential biomarkers. ET-1, a vasoactive, profibrotic and proinflammatory peptide, PIIINP, a marker of enhanced collagen turnover and fibroblast activity, and CCL2, an inflammatory cytokine, are also found to be elevated in BALF and

serum of humans with IPF (Uguccioni et al. 1995, Lammi et al. 1999, Reichenberger et al. 2001, Capelli et al. 2005). PIIINP concentrations in BALF and ET-1 concentrations in serum and BALF could also help to differentiate CIPF from its main differential diagnosis, CB (Krafft et al. 2011b, Heikkilä et al. 2013).

Proteomics, defined as a large-scale characterization of proteins expressed by a genome (Andersson and Anderson 1998), can be used to identify biomarkers and reveal disease-specific mechanisms from different biofluids (Veenstra et al. 2005, Prasse and Müller-Quernheim 2009). The main proteomic approaches used are gel-based (2DE), gel-free (mass spectrometry), and protein chip methods (Kinnula et al. 2009). The BALF proteome reflects far better the local environment in the lung than the plasma/serum proteome and is therefore an ideal material for lung proteomic studies (Wattiez and Falmagne 2005). However, up to 89% of the BALF proteome is, in health and disease, composed of high-molecular-weight plasma-derived proteins such as albumin and immunoglobulins (Govender et al. 2007). The major challenge of BALF proteomics is that these highly abundant proteins can interfere with the detection of pathologically relevant proteins present in low amounts (Kinnula et al. 2009). It is generally considered that these low-abundance proteins are produced locally by lung epithelial cells, and therefore, they are more pathologically relevant (Govender et al. 2007). Two-dimensional differential gel electrophoresis, 2D-DIGE, offers a reliable technique for quantitative analysis of complex protein samples such as BALF (Magi et al. 2006).

## **2.2 TRANSFORMING GROWTH FACTOR (TGF)- $\beta$ SUPERFAMILY**

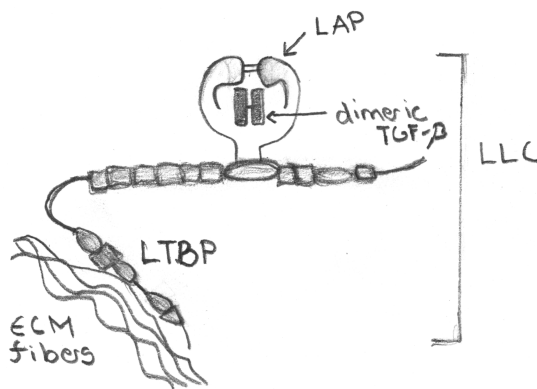
### **2.2.1 MEMBERS OF THE TGF- $\beta$ SUPERFAMILY**

The first members of the TGF- $\beta$  superfamily were discovered in the 1980s, and since then, more than 40 members of this polypeptide family have been identified (Santibañez et al. 2011). The TGF- $\beta$  superfamily can be divided to several subfamilies, including TGF- $\beta$ s, activins/inhibins, bone morphogenetic proteins, growth and differentiation factors, and Müllerian inhibitory factors. All of these family members share similar dimeric structure and participate in different aspects of cell differentiation, immune regulation, inflammation, tissue homeostasis, and embryonic development (Santibañez et al. 2011).

### 2.2.2 TGF- $\beta$ STORAGE, ACTIVATION, AND SIGNALING IN LUNGS

In mammals, three structurally related, highly conserved isoforms of TGF- $\beta$ , TGF- $\beta$  1, 2, and 3, are expressed and secreted by almost all cell types (Blobe et al. 2000, Santibañez et al. 2011). Although all three isomers are expressed by the lung, only TGF- $\beta$ 1 has been associated with fibrotic response in tissue samples from human IPF patients (Khalil et al.1996, Coker et al. 2001) and in animal models (Coker et al.1997, Ask et al. 2008).

All TGF- $\beta$ s are produced as inactive latent complexes, consisting of a mature TGF- $\beta$  and a latency-associated protein (LAP), which are unable to associate with their receptors. TGF- $\beta$  is cleaved from LAP before it is secreted by the cell, but it remains attached to LAP by non-covalent bonds forming a small latent complex. This complex then binds to latent TGF- $\beta$  binding protein (LTBP), forming a large latent TGF- $\beta$  complex, which is then secreted by the cell and bound to ECM (see Figure 2) (Taipale et al. 1994, Koli et al. 2001, 2008).

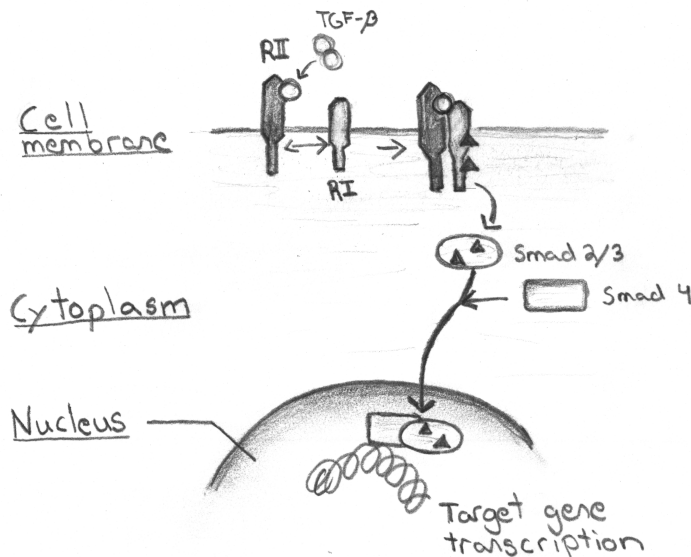


**Figure 2** A schematic picture of a large latent TGF- $\beta$  complex (LLC) consisting of dimeric TGF- $\beta$ , latency-associated protein (LAP), and latent TGF- $\beta$  binding protein (LTBP) bound to extracellular matrix (ECM) fibers (modified from Koli et al. 2008).

The latent TGF- $\beta$ -1 has been shown to be associated with the ECM via LTBP-1 (Taipale et al. 1994). LTBP-1 forms together with fibrillins the LTBP/fibrillin ECM protein family (Hyytiäinen et al. 2004). LTBP-1 has an important role in the targeting of large latent complexes to ECM, and this way also regulates the storage and the activation process of TGF- $\beta$ s (Koli et al. 2001, 2008). LTBP-1 has been found to be significantly upregulated in human IPF patient lungs (Leppäranta et al. 2012). Fibrillin-1 is the major component of connective tissue microfibrils. It has been shown to be associated with LTBP-1 and to regulate the storage and bioavailability of TGF- $\beta$ -1 (Isogai et al. 2003, Chauldry et al. 2007, Massam-Wu et al. 2010). The expression of fibrillin-2 has been shown to participate in the wound healing process, although its expression is mainly considered to be restricted to developing fetal tissues (Brinckmann et al. 2010). Aberrant activation of developmental

pathways is typical for IPF (Selman et al. 2008), and fibrillin-2 has recently been shown to be activated in adult fibrotic lungs (Leppäranta et al. 2012).

In normal tissue homeostasis, considerable amounts of TGF- $\beta$  are stored in the ECM as latent complexes. In healthy lungs, TGF- $\beta$ -1 is mostly in latent form, in contrast to fibrotic lungs, where it is active (Xu et al. 2003, Koli et al. 2008,). The activation process of TGF- $\beta$  is characterized by the release of TGF- $\beta$  from the latent complex by the action of different proteases, including plasmin and MMPs, thrombospondin, integrin, or reactive oxygen species (Annes et al. 2003). After extracellular activation, the binding of TGF- $\beta$ s to cell receptors leads to phosphorylation of signaling proteins Smad2 and Smad-3, which then bind to Smad4. The activated Smad complex moves to the nucleus, where it regulates the target gene transcription (see Figure 3) (Blobe et al. 2000, Chauldry et al. 2007). In addition, non-Smad-dependent pathways exist (Moustakas and Heldin 2005).



**Figure 3** A schematic picture of intracellular TGF- $\beta$  signaling mediated by the Smad family of proteins. Binding of TGF- $\beta$  (circles) to type II TGF- $\beta$  receptor (RII) results in RI binding to the complex and phosphorylation (indicated by triangles) of RI. This leads to phosphorylation of signal transducer proteins Smad2/3, which then bind to Smad4 and the complex moves to the nucleus (modified from Blobel et al. 2000, Shi and Massague 2003).

### 2.2.3 ROLE OF TGF- $\beta$ IN DEVELOPMENT AND TISSUE HOMEOSTASIS

TGF- $\beta$  family members have a key role in embryogenesis, where they induce epithelial-mesenchymal transition (EMT) leading to normal organogenesis (Derynck and Akhurst 2007, Selman et al. 2008). The most important

functions of TGF- $\beta$  in normal tissue homeostasis are the regulation of cell proliferation (i.e. inhibition of growth and induction of apoptosis), induction of ECM synthesis (i.e. induction of ECM proteins, such as collagen, and increasing the expression of proteinase inhibitors that degrade the ECM), and inhibition of the immune system (Hyytiäinen et al. 2004).

#### **2.2.4 TGF- $\beta$ IN FIBROTIC DISEASES**

When the balance in the interactions between the ECM and cells is disturbed, a disease can result. ECM synthesis is upregulated in the normal wound healing process, but if inappropriate activity of TGF- $\beta$  occurs, due to persistent injury or failed repair, it can lead to excessive ECM formation and fibrosis (Blobe et al. 2000, Coward et al. 2010).

Activation of the TGF- $\beta$  pathway has been shown in lungs of CIPF dogs (Krafft et al. 2012), in canine hepatic fibrosis (Spee et al. 2006), and in canine mitral valve disease (Aupperle et al. 2008).

TGF- $\beta$ 1 has profound effects on epithelial cells and fibroblasts, which are central to the pathogenesis of human pulmonary fibrosis (Coward et al. 2010). Abnormal TGF- $\beta$  regulation and function have also been linked to many other human diseases, including chronic obstructive pulmonary disease, liver cirrhosis, glomerulonephritis, atherosclerosis, and cancer (Blobe et al. 2010, Santibañez et al. 2011).

TGF- $\beta$ 1 overexpression, mainly from AEC in IPF, is responsible for fibroblast proliferation and transformation into myofibroblasts, which in turn cause the exaggerated accumulation of ECM (fibrosis) and contribute to basement membrane disruption and epithelial cell death (Coward et al. 2010, King et al. 2011, Micallef et al. 2012). TGF- $\beta$  is also the major inducer of EMT in adult tissue (Willis and Borok 2007). EMT is featured by epithelial cells losing many of their epithelial characteristics and obtaining properties of mesenchymal cells, giving rise to fibroblasts and myofibroblasts (Willis and Borok 2007). EMT has been shown to be reinstated in cancer and fibrosis processes (Derynck and Akhurst 2007, Selman et al. 2008). In addition, TGF- $\beta$ 1 is a potent inducer of lung epithelial cell apoptosis (Hagimoto et al. 2002) and promotes lung epithelial cell migration (Yu et al. 2008) and lung fibroblast collagen synthesis (Raghu et al. 1989).

#### **2.2.5 ACTIVINS**

Activins are members of the TGF- $\beta$  superfamily of cytokines. Activin A was first identified in 1980s as an inducer of follicle-stimulating hormone (FSH) secretion from the anterior pituitary (Liang et al. 1986, Vale et al. 1986). Several activin subunits have since been discovered, but biological activities of activin A, B, and AB have been described best (Hedger et al. 2011).

Published data on the role of activins in canine normal and pathological tissue processes are sparse, but activins have been reported to be involved in

regulation of the estrus cycle of the bitch (Marino et al. 2003) and to be produced by Leydig cells in adult male testis (Marino and Zanghi 2013).

Activins are greater than 97% conserved across species, implying their high individual functional importance (Hedger and de Kretser 2013). Activin A is a homodimer of two  $\beta$ A subunits, and activin B is a homodimer of two  $\beta$ B subunits. These subunits are encoded by genes named inhibin beta A (INHBA) and INHBB (Hedger and de Kretser 2013). Inhibins, in contrast, suppress FSH secretion. They are heterodimers comprising  $\beta$  and  $\alpha$  subunits, and their expression is mainly limited to gonads and to a lesser extent to the pituitary gland, placenta, adrenal gland, and central nervous system (Hedger and de Kretser 2013).

Since its discovery, activin A has been shown to be involved in a variety of inflammatory, repair, and fibrotic processes, in addition to its reproductive roles (Matsuse et al. 1996, Aoki et al. 2005, de Kretser et al. 2012). Most published studies have concentrated on the role of activin A in acute and chronic inflammatory processes, but importance of activin B in these processes has also been suggested (de Kretser et al. 2012). After binding of activins to activin-specific transmembrane type II receptors, the type I receptor, usually activin receptor-like kinase 4, phosphorylates, which leads to intracellular Smad2/3 signaling and cellular effects (Bernard et al. 2006). This Smad2/3 signaling pathway is common to TGF- $\beta$ s (Hedger and de Kretser 2013). Activin B has also been demonstrated to bind to another type I receptor, ALK-7 (Bernard et al. 2006), as well as to signal through the P-Smad1/5/8 pathway (Besson-Fournier et al. 2012). Follistatin is the major endogenous activin inhibitor, which can block the biological activities of activin (Aoki et al. 2005).

In the lung, activins have a role in the developmental processes (Zhao et al. 1996), and activin A has also been suggested to participate in the pathophysiology of human pulmonary fibrosis (Matsuse et al. 1996, de Kretser et al. 2012), asthma (Karagiannidis et al. 2006) and acute respiratory distress syndrome (ARDS) (Apostolou et al. 2012). The role of activin A in the lung fibrosis process is supported by its ability to promote proliferation of airway smooth muscle cells (Cho et al. 2003) and proliferation of lung fibroblasts and their differentiation into myofibroblasts (Ohga et al. 1996). Furthermore, follistatin has been shown to attenuate acute lung injury in the early phase and later fibrosis in a bleomycin-induced pulmonary fibrosis model in mice (Aoki et al. 2005), and an activin A neutralizing protein was found to reduce acute alveolar injury in LPS-challenged mice (Apostolou et al. 2012). Overexpression of Activin-A has been demonstrated to induce ARDS-like pathology in experimental mouse ARDS (Apostolou et al. 2012). Pathogenesis of ARDS is characterized by DAD and consists of three distinctive phases, early, proliferating, and fibrotic (Castro 2006, DeClue and Cohn 2007). ARDS is also recognized in dogs and can be secondary to various etiological factors (DeClue and Cohn 2007).

### 3 AIMS OF THE STUDY

Aims of this thesis were as follows:

1. To describe the natural course of CIPF in WHWTs and to evaluate survival, prognostic factors, and exercise intolerance in CIPF WHWTs (Study I).
2. To evaluate the bronchoalveolar lavage fluid proteome of CIPF WHWTs, dogs with chronic bronchitis, and control dogs to determine whether different protein expression profiles exist and to reveal potential biomarkers (Study II).
3. To investigate the role of TGF- $\beta$ , the major profibrotic molecule of human IPF, its extracellular matrix regulatory proteins, LTBP-1 and fibrillin2, and activins, members of the TGF- $\beta$  superfamily, in the molecular pathobiology of CIPF in WHWTs (Studies III and IV).



## **4 MATERIALS AND METHODS**

### **4.1 ETHICAL APPROVAL FOR STUDY PROTOCOLS**

The study protocols of privately owned, prospectively recruited healthy and CIPF WHWTs were approved by the Committee of Experimental Animals of Western Finland (ESHL-2008-05403/Ym-23) and the Ethics Committee for Animal Experimentation at the University of Helsinki (5B/2008) (Studies I, II, III, and IV).

The laboratory beagles (Study II) were part of a university research colony and were housed and cared for in accordance with the guidelines of the Ethics Committee for Animal Experimentation at the University of Helsinki (HY132-05).

The use of retrospectively retrieved lung samples (Studies III and IV) from the archives of the Department of Pathology and Parasitology at the Faculty of Veterinary Medicine, University of Helsinki, and the use of leftover BALF samples stored from client-owned CB dogs (Study II) at the University of Helsinki Veterinary Teaching Hospital were approved by owners for research purposes.

Human lung UIP and NSIP samples (Study III) were acquired with informed consent either from lungs explanted during lung transplantation or from diagnostic biopsies. The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital (Permission no. 426/13/03/01/09), Helsinki, Finland.

### **4.2 SAMPLE POPULATION**

Total number of animals: The study population consisted of 22 WHWTs with CIPF and 13 healthy WHWTs. In addition, samples from 5 dogs of other breeds with CB (Study II), 4 healthy beagles (Study II), 4 dogs of other breeds with ARDS (Study IV), 5 human cases with UIP, and 6 human cases with NSIP (Study III) were used.

Confirmation of disease and health in WHWTs: All CIPF WHWTs were prospectively recruited. CIPF was confirmed histopathologically in 21/22 CIPF WHWTs and by HRCT in 1/22 (still alive). For healthy WHWTs, 11/13 were prospectively recruited animals and 2/13 were retrospectively retrieved lung samples. The health status of 11 control WHWTs (Study I) was confirmed with history and thorough physical and clinical examinations. None of the control WHWTs showed signs or findings indicative of pulmonary disease and 1/11 was also in later necropsy confirmed to be healthy (lung tissue sample used in Studies III and IV). For the two retrospectively retrieved control WHWT lung samples (used in Studies III

and IV), the reasons for euthanasia were intestinal intussusception and joint disease. These two dogs had a complete necropsy examination performed, which revealed no findings, histopathological or otherwise, related to lung disease.

Study I: Dogs included in the clinical follow-up and prognostic factor study consisted of 15 CIPF WHWTs and 11 healthy WHWTs that were prospectively recruited during 2007-2012 and followed until death or study endpoint (September 2012), at the Veterinary Teaching Hospital of the University of Helsinki, Finland.

The median age of the CIPF WHWTs (7 intact males, 2 intact females, 1 neutered male, 5 neutered females) at the time of presentation was 12 (range 8-13) years. The median age of the healthy control WHWTs (3 intact males, 1 intact female, 7 neutered females) at the time of presentation was 9 (range 6-13) years.

Study II: The study material for proteomic analysis consisted of BALF samples obtained from 6 WHWTs with histopathologically confirmed CIPF, 5 dogs of other breeds that were confirmed to have CB on the basis of results of the clinical examinations performed to exclude other causes of clinical signs, and 4 clinically and later histopathologically confirmed healthy laboratory beagles.

The CIPF WHWTs were aged 11-15 (mean 12) years at the time of sample collection. The CB dogs were aged 2-11 (mean 10) years at the time of diagnosis and sample collection and were of 5 breeds (one of each of the following: Labrador Retriever, Cairn Terrier, Australian Shepherd Dog, Welsh Gorgi, and English Springer Spaniel). The Beagles were aged 5-9 (mean 7) years.

Study III: The study material for immunohistochemistry consisted of lung samples from 14 CIPF WHWTs, 3 WHWTs without lung disease, 5 human cases with UIP, and 6 human cases with NSIP. All of the canine lung samples were necropsy samples taken after euthanasia.

At the time of tissue sampling, the median age of the CIPF WHWTs was 13 (range 10-16) years. The three control WHWTs were aged 2, 6 (retrospective samples), and 13 years at the time of euthanasia. Human patients with biopsy-proven UIP were a median age of 59 (range 40-68) years and those with NSIP 54.5 (range 45-69) years.

Study IV: The sample population consisted of 4 WHWTs with CIPF and concurrent AE, 6 WHWTs with CIPF but no AE, 4 dogs of different breeds with ARDS, and 8 WHWTs without lung disease. All 4 WHWTs with CIPF and AE were euthanized due to dyspnea and histopathologically had DAD. Five of the six WHWTs with CIPF but no AE were euthanized due to non-CIPF-related causes and one due to worsening cough. None of the 6 had DAD histopathologically. All 4 ARDS dogs had histopathological changes consistent with DAD.

For immunohistochemistry, necropsy samples from 4 WHWTs with CIPF and concurrent AE, 5 WHWTs with CIPF but no AE, 4 dogs with ARDS, and

3 WHWTs without lung disease were used. At the time of tissue sampling, the median age of the CIPF dogs was 13 (range 10-15) years. The four ARDS dogs (retrospective samples) were 2.5, 3, and 4 months and one 7 years of age, and the three control WHWTs were 2, 6 (retrospective samples), and 13 years at the time of euthanasia and tissue sampling. The ARDS dogs were of 4 different breeds (one each of King Charles Spaniel, Miniature Bull Terrier, French Bulldog, and Norfolk Terrier). The suspected underlying etiology of ARDS was in two cases aspiration, in one case upper airway obstruction and in one case idiopathic ARDS.

For Western blot analysis, BALF samples from 2 WHWTs with CIPF and concurrent AE, 4 WHWTs with CIPF but no AE, and 6 healthy control WHWTs were used. BALF samples from WHWTs with CIPF were collected either immediately after euthanasia (4/6) or at the time of diagnosis (2/6). The median age of the WHWTs with CIPF at the time of sample collection was 14 (range 11-15) years. The median age of the 6 healthy control WHWTs was 8 (range 7-13) years at time of sample collection. One control sample was collected immediately after euthanasia, 5 samples at time of study inclusion.

#### **4.3 CLINICAL INFORMATION OF DISEASED AND HEALTHY WEST HIGHLAND WHITE TERRIERS (WHWTs) AND SELECTION CRITERIA FOR PROGNOSTIC PARAMETERS**

The clinical examinations of WHWTs with CIPF in the clinical follow-up study (Study I) were performed as described previously (Heikkilä et al. 2011a). Briefly, the diagnostic evaluation at the time of presentation consisted of history and physical examination (15/15), hematology and serum biochemistry (15/15), fecal examination (14/15), arterial blood gas analysis (15/15), thoracic radiography (15/15), echocardiography (13/15), high-resolution computed tomography (HRCT) (10/15), and bronchoscopy and BAL (9/15). After the initial visit, the dogs were followed in control visits at 3- to 6-month intervals and additionally as needed. At control visits, a physical examination, hematology and serum biochemistry, arterial blood gases, and thoracic radiography were performed. Diagnosis of CIPF was confirmed by histopathology in 13 deceased animals, as described in Syrjä et al. (2013), and by HRCT in two dogs still alive at the study endpoint.

The causes of death were divided for survival analysis purposes into CIPF-related (defined as euthanasia due to acute dyspnea or severe progression of respiratory signs) or non-CIPF-related. The onset of clinical signs was defined as the time-point when the owner first noticed respiratory signs or exercise intolerance.

Parameters representing possible prognostic factors predicting survival of CIPF dogs were chosen among the clinical information collected at the time of diagnosis. Only parameters that were available for at least ten diseased

animals and that had previously been shown to be altered in WHWTs with CIPF (Heikkilä et al. 2011a, Krafft et al. 2011b) were chosen. The parameters analyzed included partial pressures of oxygen (PaO<sub>2</sub>) and carbon dioxide (PaCO<sub>2</sub>) in arterial blood, alveolar-arterial oxygen gradient (P(A-a)O<sub>2</sub>), serum ET-1 concentration, severity of changes in thoracic radiographs, and HRCT Hounsfield unit values. These parameters were analyzed as previously described (Heikkilä et al. 2011a, Krafft et al. 2011b). In addition, prognostic value of rate of change in arterial blood gas (PaO<sub>2</sub>, PaCO<sub>2</sub>, and P(A-a)O<sub>2</sub>) and complete blood cell count values (hemoglobin, hematocrit, erythrocytes, mean corpuscular volume, neutrophils, lymphocytes, eosinophils, monocytes, and thrombocytes) between the first and last available measurement in CIPF-related death were analyzed. These analyses were performed for those diseased animals that had at least one control visit (12/15) before death or study endpoint.

For control WHWTs, similar clinical examinations as for WHWTs with CIPF were performed. Dogs representing the same age group as WHWTs with CIPF, middle-aged to older, were chosen. The health status of control WHWTs was confirmed with history and physical examination (11/11), hematology and serum biochemistry (11/11), fecal examination (10/11), arterial blood gas analysis (11/11), thoracic radiography (11/11), echocardiography (10/11), HRCT (8/11), and bronchoscopy and BAL (8/11). Ten dogs were included in the survival analysis. Follow-up was performed by control visits (3/10) or by contacting the owner by telephone after the study endpoint (7/10).

#### **4.4 6MWT**

To evaluate exercise tolerance in CIPF WHWTs relative to healthy WHWTs, the 6MWT was performed on 6 WHWTs with CIPF at the time of diagnosis and on 5 healthy control WHWTs (Study I). The WHWTs were part of the follow-up study population. The test was repeated in five WHWTs with CIPF during their control visits. The dogs were walked along a quiet 63.5-m straight corridor on a leash at their own pace, for six minutes. The 6MWD was recorded in meters and heart rate, body temperature, oxygen saturation (SpO<sub>2</sub>) by pulse oximetry, and arterial blood gases were measured before and after walking.

#### **4.5 BRONCHOALVEOLAR LAVAGE FLUID (BALF) COLLECTION AND SAMPLE PROCESSING**

Bronchoscopy, BAL, and sample processing (Studies II and IV) were performed as described by Heikkilä et al. (2011a). Briefly, BAL was performed during bronchoscopy in dogs anesthetized intravenously or

immediately after euthanasia. The right and left caudal lung lobes were lavaged with warm (37°C) saline (0.9% NaCl) solution (2 mL/kg, divided into 2 aliquots), and the samples were immediately processed for further analyses and then frozen at -80°C.

## 4.6 ASSAYS

### 4.6.1 TWO-DIMENSIONAL DIFFERENTIAL GEL ELECTROPHORESIS

For proteomic analysis of BALF samples from CIPF WHWTs, CB dogs, and control dogs (Study II), 2D-DIGE was performed.

The BALF samples were thawed, prepared for proteomic analysis and labeled with cyanine dyes as described in the manufacturer's protocol. The labeled samples were separated via 2D-DIGE as described elsewhere (Ünlü et al. 1997).

Immobilized pH gradient strips were used for isoelectric focusing. They were loaded with 150 µg of protein by use of a cup-loading method. Isoelectric focusing was performed (IPGPhor, GE Healthcare, Uppsala, Sweden), and after completion, second-dimension gel electrophoresis was conducted with 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. Gels were scanned between low-fluorescence glass plates (FLA-5100 laser scanner, Fujifilm, Tokyo, Japan). After gels were scanned, they were stained with silver stain without aldehyde (PlusOne silver staining kit, GE Healthcare, Uppsala, Sweden). The gel images were analyzed and statistically assessed (DeCyder, version 7.0, GE Healthcare, Uppsala, Sweden).

After image analysis, protein spots of interest were manually excised from the gels and digested in gel with trypsin (Sequencing grade modified trypsin, V5111, Promega, Madison, WI, USA), as described elsewhere (Shevchenko et al. 1996, Jensen et al. 1998). Samples were concentrated and desalinated (C18 trap column, SGE Analytical Science, Griesham, Germany), followed by mass spectrometry analysis. LC-MS/MS of peptides was performed on a hybrid quadrupole-TOFMS with a nanospray source (QSTAR Elite, Applied Biosystems, Foster City, CA, USA). Identification of proteins was performed with a local MASCOT (Matrix-Science, London, UK) search engine, against an in-house database of published canine sequences. The results were evaluated by considering the probability score, sequence coverage, and correspondence between the estimated and experimental isoelectric point and molecular weight.

### 4.6.2 IMMUNOHISTOCHEMISTRY

Selection of lung samples for immunohistochemistry of CIPF WHWTs (Studies III and IV) was done as described in Syrjä et al. (2013). In brief, one slide from the paraffin block with 1-3 lung sections having lesions most

compatible with UIP in man based on hematoxylin and eosin staining was selected for each CIPF case. For CIPF samples representing AE and for ARDS samples (Study IV), lung sections displaying DAD were selected.

Antibodies used for immunohistochemical staining in Study III were polyclonal rabbit antibody against P-Smad2/3 (Ser465/467, Millipore, Billerica, MA, USA), mouse monoclonal antibody against LTBP-1 (Mab388, R&D Systems, Minneapolis, MN, USA), and mouse monoclonal antibody against fibrillin-2 (clone 48, Millipore, Temecula, CA, USA). In Study IV, these were mouse monoclonal antibodies (mAbs) against human activin-A (mAb 18/26A) and activin-B (mAb 12/9A) subunits (encoded by INHBA and INHBB genes, respectively) generated by AnshLabs LLC (Webster, TX, USA).

Paraffin-embedded lung tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigens were retrieved by heating the sections in 0.01 M citrate buffer in a microwave oven until the solution came to a boil and then for a further 10 min. The solution was left to cool down to room temperature. For immunostaining, the Novolink Polymer Detection System (Novocastra, Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK) was used according to the manufacturer's protocol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were exposed to the primary antibody at 4°C overnight (P-Smad2 diluted 1:500, LTBP-1 diluted 1:100 and fibrillin-2 diluted 1:800, Study III) or in room temperature for 1.5 hours (activin A diluted 1:100 and activin B diluted 1:200, Study IV). The bound antibodies were visualized by using diaminobenzidine (DAB). The sections were counterstained with Mayer's hematoxylin and mounted on glass slides. The specificity of the staining was confirmed by using specific isotype controls for each antibody (Rabbit Isotype Control and Mouse Isotype Control; Invitrogen, Carlsbad, CA, USA). For all staining, control sections were negative.

#### **4.6.3 WESTERN BLOT ANALYSIS**

Verification of 2D-DIGE results (Study II) was performed with Western blot analysis. Altogether, 20 µL of each sample was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane via a semidry blotting apparatus (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% bovine serum albumin in 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20 at room temperature. Membranes were then incubated overnight at 4°C with primary antibody (anti-apolipoprotein A-1, anti-β-actin, anti-C3, or anti-haptoglobin) in Tris-buffered saline solution with Tween, which was followed the next morning by incubation with an appropriate secondary antibody conjugated with alkaline peroxidase. Colorimetric development of bound immunocomplexes was performed, and Western blot bands were photographed (AlphaImager HP system, Alpha Innotech Corp., San Leandro, CA, USA).

Detection of activin B in BALF of CIPF and healthy WHWTs was performed with Western blotting (Study IV). Recombinant activin B (R&D Systems, Minneapolis, MN, USA) was used as a positive control. BALF samples were thawed and then concentrated. SDS-PAGE electrophoresis was carried out in 4-15% polyacrylamide gradient gels (Mini-PROTEAN TGX Precast Gels, Bio-Rad, Hercules, CA, USA) using a vertical slab gel apparatus under non-reducing conditions (Mini-PROTEAN Tetra Cell, Bio-Rad). Altogether, 25 µL of each sample was separated by electrophoresis, after which proteins were transferred to a polyvinylidene difluoride membrane (Hybond-P, PVDF Transfer Membrane, GE Healthcare, Uppsala, Sweden) via a tank blotting apparatus (Mini Trans-blot®, Bio-Rad). Membranes were blocked with 10% skim milk in 0.1% Tween/TBS at room temperature. Membranes were then incubated overnight at 4°C with primary antibody (anti-activin B) in 2.5% skim milk in 0.1% Tween/TBS, followed the next morning by incubation with an appropriate secondary antibody conjugated with horseradish peroxidase (Polyclonal Goat Anti-Mouse Immunoglobulins/HRP, Dako, Denmark A/S). Bound immunocomplexes were visualized (SuperSignal West Dura Substrate, Thermo Scientific, Rockford, IL, USA), and bands were imaged using chemiluminescent scanning (LAS-3000, FUJIFILM, Tokyo, Japan).

## 4.7 STATISTICAL METHODS

Descriptive statistics are presented as median and range for all studies. All comparisons were performed as two-tailed tests. A P-value of <0.05 was considered significant.

Study I: Statistical analyses were conducted using SAS System for Windows, version 9.3 (SAS Institute Inc., Cary, NC, USA) and SPSS 20.0 for Mac (SPSS Inc., Chicago, IL, USA).

Both CIPF-specific and all-cause survival times were calculated. In the CIPF-specific analysis, dogs were censored if they were alive (2 WHWTs) or had died due to a non-IPF-related cause (6 WHWTs). For the all-cause survival analysis, dogs were only censored if they were alive. The all-cause survival of WHWTs with CIPF against the control group was compared with Kaplan-Meier (KM) survival curves and estimated together with Cox proportional hazards models. The Cox model was adjusted for the dog's age at the time of diagnosis/study inclusion.

For prognostic factor analysis, KM curves and Cox regression analysis were performed for categorical variables (radiographic values) and Cox regression for continuous variables. The survival time was calculated from onset of clinical signs. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for all models. The effects of rate of change on repeated measure parameters from the time of diagnosis to the last available measurement on CIPF-related death were assessed with logistic regression

models. Odds ratios (ORs) with 95% CIs were calculated to quantify the results.

For 6MWT results, Mann-Whitney U-test was used to compare differences between control and diseased animals, and Kendall's tau-b correlation coefficient was used for correlations. Wilcoxon signed-rank test was used for all paired samples in 6MWT and for comparisons between initial and last available clinical measurements.

Study II: 2D-DIGE gel images were analyzed and statistically assessed (DeCyder, version 7.0, GE Healthcare, Uppsala, Sweden). Protein spots that had a minimum 1.5-fold difference in mean spot volume ratios in all biological replicates were tested via an ANOVA. If differences were detected, the Student's t test was applied to compare differences in data between healthy control dogs and dogs with IPF and between healthy control dogs and dogs with CB. Proteins with a value of  $P < 0.05$  were selected for identification.

Studies III and IV: Semiquantitative immunohistochemical staining data were analyzed with SPSS 20.0 for Mac (SPSS, Chicago, IL, USA). Pairwise comparisons for Study III groups were performed using non-parametric Mann-Whitney U-test. Comparisons between study groups in Study IV were performed using non-parametric Kruskal-Wallis with subsequent pairwise comparisons using the Dunn test. Kendall's tau-b correlation coefficient was used for correlations (Study III).



## 5 RESULTS

### 5.1 CLINICAL FINDINGS DURING DISEASE PROGRESSION AND CAUSE OF DEATH AND RELATED HISTOPATHOLOGIC FINDINGS

Of the 15 WHWTs with CIPF included in the follow-up study (Study I), 12 had at least one control visit before death or study endpoint. A significant decrease occurred between the first (median 58.9 mmHg, range 50.6–64.0 mmHg) and the last (median 50.0 mmHg, range 44.3–51.5 mmHg) PaO<sub>2</sub> values ( $P = 0.04$ ) and a significant increase between the first (median 54.8 mmHg, range 43.5–67.4 mmHg) and the last (median 70.9 mmHg, range 57.6–80.8 mmHg) P(A-a)O<sub>2</sub> measurement ( $P = 0.04$ ) in all 5 CIPF WHWTs that had died of CIPF-related causes. During the disease course temporary improvements were noted in PaO<sub>2</sub> values in 7/12 WHWTs with CIPF. The magnitude of these improvements varied from 2.8 mmHg to 19.9 mmHg, with a median of 7.9 mmHg. No significant change was noted in body weight of 12 WHWTs with CIPF between the first (median 9.9 kg, range 7.8–11.9 kg) and last (median 9.6 kg, range 7.2–11.8 kg) measurement ( $P = 0.31$ ) or between the first (median 50%, range 44–61%) and last (median 51%, range 35–64%) hematocrit values ( $P = 0.18$ ). The bronchointerstitial radiographic changes were initially classified as severe in 7/12 animals or moderate in 5/12 animals. In 6 animals, no change in pattern severity was seen, in 3 the classification changed from moderate to severe, and in 3 it changed from severe to moderate during disease progression. Three animals had an alveolar pattern at the time of diagnosis, but not at the last control visit, and 5 dogs with no alveolar pattern at the first visit had it at the last control visit.

Seven of 13 deceased WHWTs with CIPF died of CIPF-related causes. Five of these 7 dogs were euthanized due to dyspnea and 2 due to severe progression of respiratory signs. Four of the 5 dyspneic dogs showed acute alveolar damage (DAD) histopathologically. Causes of death or euthanasia in the non-CIPF-related death group (6 dogs) were pyelonephritis, metastatic mammary tumor, drowning, acute uremia, acute vomiting, and hemoabdomen. The dog that drowned also had DAD in the lungs. Three of the control WHWTs were euthanized before the study endpoint. The reasons were dementia in one dog, mammary tumor in another, and unknown in the third. None of the control WHWTs had any owner-reported respiratory signs before death or study endpoint.

## 5.2 SURVIVAL AND PROGNOSTIC FACTORS

The median follow-up time of 15 CIPF WHWTs included in the follow-up study (Study I) was 15 (range 0–40) months, and the median follow-up time of 10 control WHWTs was 35 (range 6–55) months.

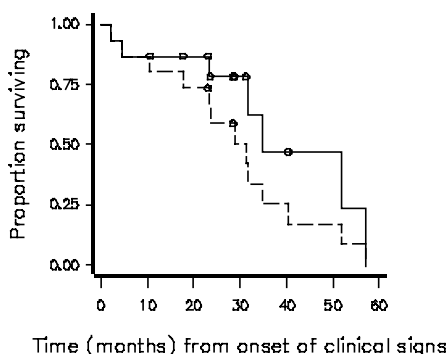
Thirteen of 15 CIPF WHWTs were euthanized during the study period and 2 were still alive at the study endpoint. The median all-cause and CIPF-specific survival times of deceased WHWTs from birth, from onset of clinical signs, and from time of diagnosis are presented in Table 2. All-cause survival refers to death or euthanasia due to CIPF and non-CIPF-related causes together and CIPF-specific survival to death or euthanasia due to only CIPF-related causes. The median survival of deceased control WHWTs (3/10) was 172 (range 154–184) months from birth.

**Table 2** All-cause and CIPF-specific survival times of 13 deceased CIPF WHWTs.

	All-cause survival n=13/13 (median and range)	CIPF-specific survival n=7/13 (median and range)
From birth (months)	160 (123-188)	163 (155-170)
From onset of clinical signs (months)	27 (2-51)	32 (2-51)
From diagnosis (months)	13 (0-40)	11 (0-40)

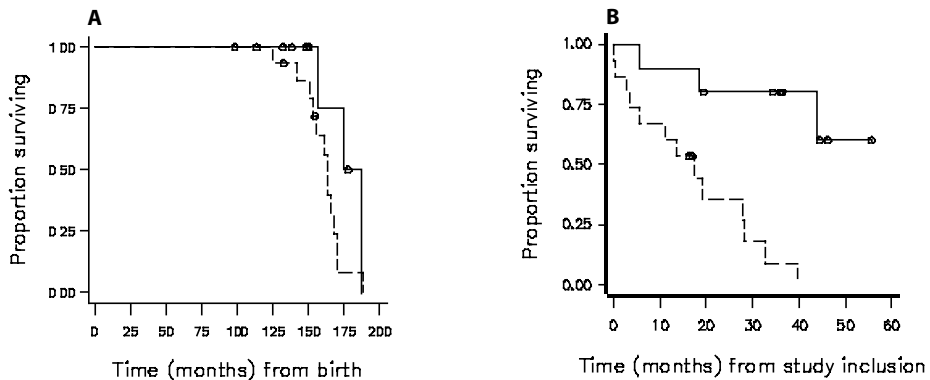
CIPF, canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier.

The KM curves for all-cause and CIPF-specific survival from onset of clinical signs are presented in Figure 4.



**Figure 4** Kaplan-Meier survival curves for all-cause survival (dotted line) and canine idiopathic pulmonary fibrosis (CIPF)-specific survival (solid line) of West Highland White Terriers (WHWTs) with CIPF from onset of clinical signs. Censored animals (for all-cause survival, WHWTs alive at study endpoint; for CIPF-specific survival, WHWTs alive and those who died of non-CIPF-related causes) are presented as circles.

Based on the Cox regression model for all-cause survival adjusted for dog's age at study inclusion, the HR for risk of death in WHWTs with CIPF from birth relative to control WHWTs was 4.6 (95% CI 1.05–19.74,  $P=0.04$ ) and from study inclusion 4.4 (95% CI 0.94–20.5,  $P=0.06$ ). The HR for age in the multivariate Cox model from birth was 0.66 (95% CI 0.42–1.04,  $P=0.08$ ) and from study inclusion 1.93 (95% CI 1.25–2.98,  $P=0.003$ ). KM curves for survival of WHWTs with CIPF and control WHWTs from birth and from study inclusion are presented in Figure 5.



**Figure 5** (A). Kaplan-Meier (KM) survival curves for all-cause survival of West Highland White Terriers (WHWTs) with canine idiopathic pulmonary fibrosis (CIPF) (dotted line), and control WHWTs (solid line) from birth. Censored animals (WHWTs alive at study endpoint) are presented as circles. (B). KM survival curves for all-cause survival of WHWTs with CIPF and control WHWTs from study inclusion.

No statistically significant prognostic factors were identified (Table 3). In addition, no significant effects of the rate of change in repeated-measure variables on the risk of CIPF-related death were detected (Table 4).

**Table 3** *Cox regression analysis of the effects of prognostic variables on CIPF-specific/all-cause survival in WHWTs with CIPF.*

Variable (unit change)	N	CIPF-specific survival HR (95% CI)	P	All-cause survival HR (95% CI)	P
PaO <sub>2</sub> mmHg (10)	15	0.60 (0.21-1.72)	0.34	1.20 (0.58-2.46)	0.63
PaCO <sub>2</sub> mmHg (1)	15	0.96 (0.75-1.23)	0.75	1.02 (0.86-1.21)	0.78
P(A-a)O <sub>2</sub> mmHg (10)	14	1.59 (0.67-3.73)	0.29	0.93 (0.51-1.68)	0.81
ET-1 pg/mL (1)	10	1.11 (0.70-1.75)	0.67	0.91 (0.59-1.42)	0.69
HU (100)	10	0.71 (0.08-5.98)	0.75	0.69 (0.27-1.76)	0.44
Severity of radiographic bronchointerstitial pattern (severe)	15	2.68 (0.29-24.4)	0.38	0.66 (0.20-2.21)	0.50
Presence of radiographic alveolar pattern (yes)	15	1.87 (0.36-9.74)	0.46	1.51 (0.46-4.95)	0.50

CIPF, canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier; HR, hazard ratio; CI, confidence interval; PaO<sub>2</sub>, partial pressure of oxygen in arterial blood; PaCO<sub>2</sub>, partial pressure of carbon dioxide in arterial blood; P(A-a)O<sub>2</sub>, alveolar-arterial oxygen gradient; ET-1, serum endothelin-1 concentration; HU, Hounsfield unit (describing overall density of lung parenchyma in high-resolution computed tomography image).

**Table 4** *Logistic regression analysis of the effects of change over time in repeated-measure parameters on CIPF-specific death in 12 WHWTs with CIPF.*

Parameter (unit change)	OR	95% CI
PaO <sub>2</sub> mmHg (1)	0.85	0.62-1.15
PaCO <sub>2</sub> mmHg (1)	0.88	0.62-1.24
P(A-a)O <sub>2</sub> mmHg (1)	1.15	0.88-1.49
Hematocrit % (1)	1.37	0.78-2.34
Erythrocytes 10 <sup>12</sup> /L (0.1)	1.21	0.89-1.65
Hemoglobin g/L (1)	1.12	0.91-1.40
MCV fL (1)	0.61	0.28-1.36
Neutrophils 10 <sup>9</sup> /L (1)	1.13	0.82-1.54
Lymphocytes 10 <sup>9</sup> /L (0.1)	0.96	0.80-1.15
Eosinophils 10 <sup>9</sup> /L (0.1)	0.72	0.38-1.34
Monocytes 10 <sup>9</sup> /L (0.1)	0.97	0.64-1.48
Thrombocytes 10 <sup>9</sup> /L (10)	0.90	0.76-1.07

CIPF, canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier; OR, odds ratio; CI, confidence interval; PaO<sub>2</sub>, partial pressure of oxygen in arterial blood; PaCO<sub>2</sub>, partial pressure of carbon dioxide in arterial blood; P(A-a)O<sub>2</sub>, alveolar-arterial oxygen gradient; MCV, mean corpuscular volume.

### 5.3 6MWT

The 6MWD and the variables measured before and after walking in CIPF and control WHWTs (Study I) are presented in Table 5.

**Table 5** *Pre-and post-walking 6MWT characteristics in six WHWTs with CIPF and in five control WHWTs, given as median and range.*

Characteristic	CIPF WHWT	Control WHWT	P
Pre-HR( bpm)	107 (80-120)	114 (92-144)	0.13
Post-HR( bpm)	108 (96-146)	126 (112-144)	0.18
	<i>P= 0.04</i>	<i>P= 0.32</i>	
Pre-body T (°C)	38.7 (38.5-39.9)	39.0 (38.0-39.1)	0.93
Post-body T (°C)	38.7 (38.5-39.2)	38.7 (38.3-39.2)	0.84
	<i>P= 0.47</i>	<i>P= 0.58</i>	
Pre-SpO <sub>2</sub> (%)	96 (87-100)	98 (96-98)	0.13
Post-SpO <sub>2</sub> (%)	97 (86-100)	98 (93-99)	0.79
	<i>P= 0.49</i>	<i>P= 0.46</i>	
Pre-PaO <sub>2</sub> (mmHg)	56.7 (49.4-64.9)	101.8 (82.0-108.0)	<b>0.01</b>
Post-PaO <sub>2</sub> (mmHg)	55.8 (37.7-61.8)	86.3 (82.0-110.0)	<b>0.01</b>
	<i>P= 0.08</i>	<i>P= 0.47</i>	
Pre-PaCO <sub>2</sub> (mmHg)	31.8 (26.1-36.9)	30.4 (27.4-34.0)	1.00
Post-PaCO <sub>2</sub> (mmHg)	28.5 (25.3-34.5)	32.7 (27.8-33.9)	0.35
	<i>P= 0.17</i>	<i>P= 0.14</i>	
Pre-P(A-a)O <sub>2</sub> (mmHg)	55.7 (45.3-70.0)	14.5 (12.2-23.8)	<b>0.01</b>
Post-P(A-a)O <sub>2</sub> (mmHg)	61.2 (53.8-82.4)	22.6 (7.8-35.3)	<b>0.01</b>
	<i>P= 0.08</i>	<i>P= 0.47</i>	
6MWD (m)	398 (273-519)	492 (420-568)	<b>0.05</b>

6MWT, six-minute walk test; CIPF, canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier; HR, heart rate; SpO<sub>2</sub>, oxygen saturation measured by pulse oximetry; PaO<sub>2</sub>, partial pressure of oxygen in arterial blood; PaCO<sub>2</sub>, partial pressure of carbon dioxide in arterial blood; P(A-a)O<sub>2</sub>, alveolar-arterial oxygen gradient; 6MWD, six-minute walk distance. Comparisons between diseased and healthy groups using Mann-Whitney U-test; paired pre- and post-walking comparisons using Wilcoxon test.

In diseased animals, PaO<sub>2</sub> seemed to have a moderate positive correlation with 6MWD, although only statistically significant at 10% (Kendall's tau-b = 0.69, *P* = 0.06). Nine to 11 months after the first 6MWT, 4 of the 5 WHWTs with IPF had a reduced 6MWD relative to their initial visit. However, the difference between 6MWD at the first visit (median 399 m, range 309–519 m) and 6MWD at the control visit (median 375 m, 230–478 m) was significant only at 10% (*P* = 0.08).

## 5.4 BALF PROTEOMIC ANALYSIS

In 2D-DIGE analysis (Study II), 18 protein spots were detected as differentially expressed in BALF of control dogs relative to expression in both groups of diseased dogs (CB dogs and CIPF WHWTs). In addition, one protein spot was significantly differentially expressed only between CIPF and healthy control dogs. For identification and further analysis, 9 protein spots

that were upregulated and 4 that were downregulated in both groups of diseased dogs and the one protein spot upregulated in the CIPF dogs were chosen based on their appearance in silver stained gel. Of the 9 proteins for which expression was upregulated, 5 were identified as complement C3, alpha-1-antitrypsin, apolipoprotein A-1, haptoglobin, and transketolase. The one protein for which expression was upregulated in the CIPF dogs was identified as  $\beta$ -actin. For the 4 proteins for which expression was downregulated, lysozyme C was identified. Verification of 2D-DIGE results was performed with Western blot analysis. Expression of  $\beta$ -actin was also detected in dogs with CB during the verification analysis. Therefore, further analysis with 2-D Western blots was performed, revealing that the expression pattern of  $\beta$ -actin protein among the dogs with CB was slightly different among individual dogs.

## 5.5 P-SMAD2, LTBP-1, AND FIBRILLIN-2 IMMUNOREACTIVITY IN FIBROTIC LUNGS

A summary of immunostaining results of proteins linked to TGF- $\beta$  signaling activity (P-Smad2), TGF- $\beta$  storage (LTBP-1), and extracellular matrix regulation (fibrillin-2) in control WHWT, CIPF WHWT, human IPF/UIP, and human NSIP lungs (Study III) is presented in Table 6.

**Table 6** *Localization and intensity of P-Smad2, LTBP-1 and fibrillin-2 immunoreactivity in control WHWT, CIPF WHWT, and human UIP and NSIP lungs.*

	P-Smad2		LTBP-1		Fibrillin-2	
	Alveolar area	Bronchial epithelium	Alveolar area	Peribroncho-vascular	Alveolar area	Peribroncho-vascular
Control WHWT	+/-	+++	+/-	+/-	+/-	++
CIPF WHWT	+++	+++	+	++	+	++
UIP	+++	+++	+++	++	++	++
NSIP	+++	+++	+	++	+	++

WHWT, West Highland White Terrier; CIPF, canine idiopathic pulmonary fibrosis; UIP, human usual interstitial pneumonia; NSIP, human non-specific interstitial pneumonia. Immunoreactivity score: -, absent; +/-, absent or very mild; +, mild; ++, moderate; +++, intense immunostaining.

### 5.5.1 P-SMAD2 IMMUNOREACTIVITY AND CORRELATION OF THE PERCENTAGE OF P-SMAD2- POSITIVE NUCLEI WITH ARTERIAL PARTIAL PRESSURE OF OXYGEN LEVELS

In CIPF samples, a high amount of P-Smad2 immunoreactivity was mainly observed in the altered alveolar epithelium. In healthy control WHWTs, only low amounts of immunoreactivity were seen in the alveolar wall cells (Table 6). Bronchial epithelial cells in all groups showed a high amount of positivity

(Table 6). In human NSIP samples, P-Smad2 immunoreactivity was observed in the altered alveolar epithelium lining the alveolar wall (Table 6) and in mononuclear inflammatory cells of the thickened alveolar and peribronchovascular interstitium. In human UIP samples, as previously reported (Khalil et al. 1996, Leppäranta et al. 2012), strong TGF- $\beta$  signaling activity, indicated by intense P-Smad2 immunoreactivity, was seen in hyperplastic epithelial cells overlying the fibroblastic foci and in parenchyma.

P-Smad2 immunoreactivity (calculated as the percentage of positive alveolar cell nuclei in the alveolar area) was significantly ( $P= 0.01$ ) increased in WHWTs with CIPF (median 71%, range 40-82%) relative to healthy WHWTs (13%, 2-17%). No differences were seen between human UIP and NSIP groups ( $P= 0.14$ ). A moderate negative correlation existed between PaO<sub>2</sub> values and P-Smad2 immunoreactivity in WHWTs with CIPF ( $P= 0.095$ ,  $\tau= 0.44$ ).

### **5.5.2 LOCALIZATION AND INTENSITY OF LTBP-1 IMMUNOREACTIVITY**

In WHWTs with CIPF, moderate granular immunoreactivity was observed in altered alveolar epithelial cells and mild multifocal staining in the alveolar interstitium. In addition, moderate granular staining in bronchial epithelial cells and mild staining in smooth muscle cells of bronchiolar and vascular walls were present in diseased WHWTs (staining of alveolar and peribronchovascular areas was quantified separately) (Table 6). In healthy WHWTs, only very mild or absent alveolar staining and very mild peribronchovascular staining were observed (Table 6). In NSIP, mild to moderate LTBP-1 immunoreactivity was noted in alveolar interstitial and peribronchovascular areas (Table 6). Staining of altered alveolar epithelium in NSIP samples was less prominent than the intense immunostaining seen in UIP samples. In UIP, as previously reported (Leppäranta et al. 2012), moderate immunoreactivity was also seen in fibroblastic foci and the surrounding parenchymal tissue.

A significant increase ( $P= 0.02$ ) in intensity of LTBP-1 immunoreactivity in peribronchovascular areas was seen in WHWTs with CIPF (median 1, range 0.5-2) relative to healthy WHWTs (0.5, 0.25-0.5). No statistical difference emerged in alveolar staining between WHWTs with CIPF and healthy WHWTs ( $P= 0.31$ ). Between UIP and NSIP groups, no differences were detected in alveolar ( $P= 0.10$ ) or peribronchovascular immunoreactivity ( $P= 0.26$ ).

### **5.5.3 LOCALIZATION AND INTENSITY OF FIBRILLIN-2 IMMUNOREACTIVITY**

Fibrillin-2 localized most intensively in peribronchial and perivascular areas and also multifocally in the alveolar interstitium of CIPF WHWT lung

samples. Absent or very mild alveolar immunoreactivity was seen in healthy WHWTs (Table 6). The alveolar immunoreactivity seen in CIPF WHWTs was generally less intense than in human UIP and NSIP samples. In NSIP, mild multifocal alveolar interstitial immunoreactivity was noted (Table 6). In UIP, mild to moderate fibrillin-2 immunoreactivity was observed in the thickened parenchymal tissue, especially in fibroblastic foci, as reported previously (Leppäranta et al. 2012). Peribronchovascular staining was present at a similar intensity in all sample groups (Table 6).

No statistical differences were detected in fibrillin-2 alveolar immunoreactivity between control WHWTs and CIPF WHWTs ( $P = 0.24$ ) or between UIP and NSIP groups ( $P = 0.77$ ), nor was a difference observed in peribronchovascular immunoreactivity ( $P = 0.65$  and  $P = 0.63$ , respectively).

## 5.6 ACTIVIN A AND ACTIVIN B IMMUNOREACTIVITY IN FIBROTIC LUNGS AND DIFFUSE ALVEOLAR DAMAGE

A summary of activin A and B immunostaining results in control WHWT, CIPF WHWT, and ARDS dog lungs (Study IV) is presented in Table 7.

**Table 7** *Localization and intensity of activin A and activin B immunoreactivity in lung tissue of control WHWTs, CIPF WHWTs, CIPF WHWTs with AE/DAD, and ARDS/DAD dogs.*

	AEC I		AEC II		Mononuclear inflamm. cells		Bronchial epithelium		Alveolar edema	
	act A	act B	act A	act B	act A	act B	act A	act B	act A	act B
Control WHWTs	-	+/-	+/-	+/-	+	+	+	+++	n/a	n/a
CIPF WHWTs	-	+	-	+++	+	+	+/-	+++	n/a	n/a
CIPF WHWTs with AE/DAD	-	++	+/-	+++	+	+	+	+++	+	++
ARDS/DAD dogs	-	++	-	+++	+	++	+	+++	++	+++

act A, activin A; act B, activin B; CIPF, canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier; AE, acute exacerbation; DAD, diffuse alveolar damage; ARDS, acute respiratory distress syndrome; AECI, alveolar epithelial cell with appearance of type I cell; AECII, alveolar epithelial cell with appearance of type II cell. Immunoreactivity score: -, absent; +/-, absent or very mild; +, mild; ++, moderate; +++, intense immunostaining; n/a, not applicable.

### 5.6.1 ACTIVIN A LOCALIZATION AND INTENSITY OF IMMUNOREACTIVITY

Absent or very mild activin A immunoreactivity was observed in the altered alveolar epithelium in CIPF samples or on the alveolar wall surface of control lung samples (Table 7). Mild positive immunostaining in bronchial epithelial cells and in mononuclear inflammatory cells was seen in less than half of the CIPF samples, comparable with findings in control lung samples. The regions



of alveolar edema seen in CIPF samples with AE also showed mild positive immunostaining for activin A (Table 7). In ARDS lungs, the alveolar epithelial lining and the activated alveolar epithelial cells were negative. Mild positive immunoreactivity was seen in mononuclear inflammatory cells and in the bronchial epithelium, and moderate positive staining of alveolar edema was observed (Table 7).

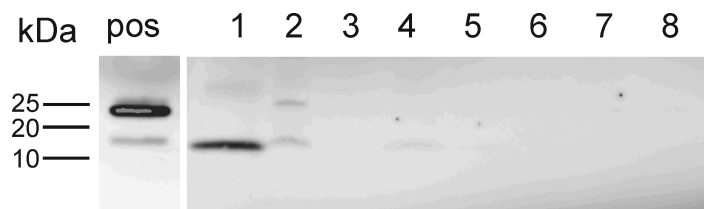
No significant difference between groups was detected in immunostaining score of AEC type I cells ( $P= 1.00$ ), AEC type II cells ( $P= 0.48$ ), mononuclear inflammatory cells ( $P= 0.27$ ), bronchial epithelium ( $P= 0.43$ ), or alveolar edema ( $P=0.08$ ).

### **5.6.2 ACTIVIN B LOCALIZATION AND INTENSITY OF IMMUNOREACTIVITY AND WESTERN BLOT ANALYSIS**

In control animals, intense activin B immunoreactivity was observed in the bronchial epithelium, whereas alveolar surface cells remained negative (Table 7). In WHWTs with CIPF, intense immunoreactivity was observed in pathologically altered AECII cells and in bronchial epithelial cells (Table 7). In WHWTs with CIPF and concurrent AE, also the alveolar edema showed intense fine granular staining. In the CIPF and control groups, mild immunoreactivity was observed in mononuclear inflammatory cells (Table 7). In ARDS lungs, intense activin B immunoreactivity was seen in altered AECII cells of samples representing the proliferative phase of ARDS and on the surface of denuded alveolar walls, alveolar edema, and hyaline membranes of samples representing the exudative phase.

A significant increase in AECII staining intensity score was seen in lungs of WHWTs with CIPF (median 3, range 2.5- 3) relative to healthy WHWTs (0.5, 0- 0.5) ( $P=0.04$ ) and in lungs of WHWTs with CIPF and concurrent AE (3, 3-3) relative to healthy WHWTs ( $P=0.02$ ). No significant difference was seen in AECII immunoreactivity between ARDS (3, 2- 3) and healthy dogs ( $P= 0.23$ ) or between CIPF with AE and ARDS dogs ( $P= 1.00$ ). No significant difference between study groups was seen in AECI ( $P= 0.08$ ), mononuclear inflammatory cells ( $P= 0.11$ ), bronchial epithelium ( $P= 1.00$ ), or alveolar edema ( $P= 0.46$ ) immunostaining results.

Activin B was detected by Western blot analysis in 2/2 BALF samples from WHWTs with CIPF and concurrent AE. In addition, a weak band was observed in 2/4 BALF samples from WHWTs with CIPF but no AE. None of the six BALF samples of control WHWTs had detectable levels of activin B. Monomeric activin B (~15 kDa) was mainly detected in the BALF samples due to the dissociation of the dimeric form of activin B (~ 30 kDa). A representative image of the Western blot results is shown in Figure 6.



**Figure 6** Western blot results of protein expression of activin B monomer of ~ 15 kDa in bronchoalveolar lavage fluid of West Highland White Terriers (WHWTs) with canine idiopathic pulmonary fibrosis (CIPF) and concurrent acute exacerbation (AE) and diffuse alveolar damage (DAD), lines 1 and 2; WHWTs with CIPF but no AE/DAD, lines 3, 4, and 5; and healthy control WHWTs, lines 6, 7, and 8. As a positive control (POS), recombinant activin B was used.

## 6 DISCUSSION

### 6.1 CLINICAL COURSE AND SURVIVAL

CIPF is a disease occurring in older WHWTs, and the magnitude of its effect on lifespan as well as the natural history of the disease had been previously unclear (Corcoran et al. 1999), as no prospective follow-up studies with controls had been published. In our follow-up study (Study I), we demonstrated that CIPF has significant negative impact on life expectancy of affected WHWTs. This finding was supported by both of our age-adjusted survival models, with risk for death from birth being 4.6 times higher and from study inclusion 4.4 times higher in diseased animals than in controls, although the risk in the latter model was statistically significant only at 10%.

The median survival after diagnosis in WHWTs with CIPF was 1 year, but some dogs lived up to 3 years (Study I). In humans, the median survival after diagnosis of IPF is 2-3 years (Raghu et al. 2011) and after diagnosis of fibrotic NSIP 6-13.5 years (Kim et al. 2006). The median survival time from onset of clinical signs in the CIPF-related death group was quite high, 2.7 years, but varied greatly, from only 2 months to 4.3 years (Study I). This variation indicates that CIPF in WHWTs may have either a rapid or a slow disease progression, as also seen in human IPF (Kim et al. 2006). Five WHWTs with CIPF in our follow-up study were euthanized due to acute dyspnea. In human IPF, these acute respiratory deteriorations of unidentifiable cause are termed AEs of IPF (Collard et al. 2007). The AE term is suitable also for CIPF, as necropsy of these dogs did not reveal an alternative explanation for acute dyspnea and 4/5 of these dyspneic dogs had DAD in lungs, which is reported in humans as a common terminal histopathological finding related to AEs of IPF (Rice et al. 2003). Despite intensive care, mortality of human AE patients is up to 80% (Kim et al. 2006). For ethical reasons and because of the expected poor outcome, benefits of treatment efforts should be carefully considered in CIPF dogs experiencing AE.

Since chronic hypoxemia can cause erythrocytosis, and weight loss could occur due to poor condition, we followed the hematocrit values and weight of the CIPF WHWTs during disease progression. Neither weight loss nor change in hematocrit was seen (Study I). Severe malnutrition is also not a feature of human IPF (Nathan et al. 2013).

Thoracic radiograph features varied during the disease independently of clinical signs and were not useful in evaluating disease progression (Study I). In humans, chest radiographs also correlate poorly with disease severity (Kim et al. 2006). In addition, evaluation of other emerging diseases in thoracic radiographs of CIPF WHWTs, such as infections or neoplasms, could be challenging, as changes seen in radiographs already at the time of diagnosis were so marked. Although none of the deceased WHWTs with

CIPF in our study had primary lung tumors detected postmortem, in man bronchogenic carcinoma occurs with increased frequency in lungs affected by IPF (Ley et al. 2011), and pulmonary neoplasms are also reported to occur in cats with an IPF-like condition (Cohn et al. 2004, Williams et al. 2004).

During the course of the disease, a declining trend in PaO<sub>2</sub> values of CIPF WHWTs was detected (Study I). The decrease between the first and last PaO<sub>2</sub> values in dogs that died of CIPF-related causes was significant, as was the increase in P(A-a)O<sub>2</sub> values. However, in some CIPF WHWTs, temporary increases in arterial oxygenation were noted, and some owners described a temporal improvement in clinical signs. As our study design did not allow systematic evaluation of the treatment effect on survival or on clinical signs due to individual treatment protocols, the impact of medication on these improvements cannot be assessed. However, human IPF is unresponsive to the treatments used in our study (Raghu et al. 2011), while patients with NSIP usually benefit from corticosteroids (Park et al. 2009). Although repeated measurements of arterial blood gas values proved to be the most valuable tool for evaluating disease progression in CIPF WHWTs, some animals with very low PaO<sub>2</sub> values survived surprisingly long due to excellent adaptation and slow deterioration in oxygenation capacity.

## **6.2 6MWT**

Exercise intolerance is one of the main clinical signs of IPF in both dogs (Heikkilä et al. 2011a) and humans (Raghu et al. 2011). However, objective evaluation of exercise intolerance in older non-athletic pet dogs can be difficult. Therefore, a noninvasive 6MWT with age- and breed-matched controls for more comparable results of exercise performance was applied (Study I). In human medicine, from the clinical perspective, the advantages of 6MWT are that it can be performed with no special equipment or training and all but the most severely ill patients are able to perform it (ATS 2002, du Bois et al. 2011). For the same reasons, 6MWT is promising for veterinary use, especially as dogs might need hours of training to walk on a treadmill and some might not cooperate at all (Kittleson et al. 1996). However, low 6MWD itself is non-specific and non-diagnostic, and thorough clinical examinations are warranted to identify the cause of the impairment (ATS 2002). In our study, we showed that 6MWT was easy to perform and well-tolerated in WHWTs with CIPF. The 6MWD in diseased animals was reduced relative to that in control dogs. In addition, the 6MWD in CIPF WHWTs was reduced at the 9- to 11-month control visit, albeit significant only at 10%. The PaO<sub>2</sub> seemed to correlate positively with 6MWD, and therefore, the 6MWD could serve as a noninvasive means of monitoring lung function in WHWTs with CIPF. However, this finding requires confirmation in larger sample sets. In pre- and postwalking variables, the only significant change was a higher postwalking heart rate in WHWTs with CIPF. The short delay when taking

the postwalking arterial sample may influence the arterial blood sample results. We did not detect a significant difference in SpO<sub>2</sub> between controls and WHWTs with CIPF, nor did we find significant postwalking oxygen desaturation. Previous studies have reported a difference in oxygen saturation between healthy dogs and dogs with pulmonary disease (Swimmer and Rozanski 2011) as well as post-walking oxygen desaturation in a group of old healthy beagles (Gault et al. 2010). This discrepancy in results may be due to different breed or device; in our study, pulse oximeter seemed to be a rather unreliable indicator of a decline in PaO<sub>2</sub> in WHWTs with CIPF. In human patients with IPF, desaturation during the test distance walked and 6MWD decline over time have been linked to a higher risk of death (Lama et al. 2003, Caminati et al. 2009, du Bois et al. 2011). Due to our low sample size, we were unable to use these parameters in prognostic factor analysis.

### **6.3 EVALUATION OF POTENTIAL PROGNOSTIC FACTORS AND BIOMARKERS**

For CIPF, no prognostic factors of survival are currently known and no biomarkers are in clinical use. A noninvasive biomarker from BALF or serum would be highly beneficial for more practical CIPF diagnosis, for evaluating disease severity and progression, and for differentiation of CIPF from CB. For human IPF, several individual predictors of survival have been reported during the last decades. These include clinical predictors, such as age, medical imaging predictors, physiologic predictors, pathological predictors, and biomarker predictors from blood and BALF (Ley et al. 2011). However, a clinically useful predictor model for human IPF is lacking (Ley et al. 2011). In our study, we aimed to evaluate potential prognostic factors affecting the survival of diseased dogs (Study I) and to identify potential biomarkers for CIPF from BALF by comparing BALF protein expressions in CIPF, CB, and healthy dogs (Study II).

No significant prognostic factors for CIPF were identified among the chosen variables, which is most likely due to our low sample size. However, a slight indication of high PaO<sub>2</sub> having a protective effect on survival was noted, supporting the use of arterial blood gas measurements in CIPF follow-up. In addition, a slight indication of high P(A-a)O<sub>2</sub> being a risk factor emerged. In humans, an increase in P(A-a)O<sub>2</sub> is associated with earlier IPF mortality (King et al. 2005). No significant effects of change in repeated measure parameters on the risk of CIPF-related death were noted. However, a slight indication of a higher risk of CIPF-related death could be seen with an increase in hematocrit, hemoglobin, and erythrocyte values. Some potentially interesting prognostic factors, including 6MWD, BALF cell counts (Heikkilä et al. 2011a), BALF PIIINP concentrations (Heikkilä et al. 2012), and pulmonary hypertension (Schober and Baade 2006), which have been shown to be altered in CIPF WHWTs and to have prognostic value in human

IPF (Lammi et al. 1999, Caminati et al. 2008, Kinder et al. 2008, Andersen et al. 2012), could not be included in our study due to low sample size.

The search for biomarkers from complex biological samples, such as BALF, is challenging, as highly abundant blood-derived proteins interfere with the detection of locally produced and more pathologically relevant proteins present in lower amounts (Govender et al. 2007, Kinnula et al. 2009). To obtain quantitative data on differences in the BALF proteome between CIPF, CB, and healthy dogs, we applied the 2D-DIGE method. However, no CIPF specific biomarkers were found. This probably reflects the difficulty in detecting the low-abundance fraction of the BALF proteome, where the proteins of interest in pulmonary fibrosis are likely to be found (Govender et al. 2007). We did, however, detect five proteins that were upregulated and one that was downregulated in CIPF and CB dogs relative to controls. In addition, one protein,  $\beta$ -actin, was initially recognized to be upregulated only in the CIPF group, but further analysis revealed that the protein was heterogeneously expressed in each of the CB dogs, and therefore, not detected in 2D-DIGE. Thus, the extent of expression of  $\beta$ -actin in CB dogs remains unknown. Of the seven differentially expressed proteins,  $\beta$ -actin is part of the cellular cytoskeleton, complement C3 is part of the complement system,  $\alpha$ -1-antitrypsin, apolipoprotein A-1, and haptoglobin are anti-inflammatory proteins, transketolase is involved in glucose metabolism, and lysozyme is part of the innate immune system.  $\beta$ -actin is one of the most frequently differentially expressed proteins found in various proteomic analyses (Petrak et al. 2008), reflecting more its involvement in the common stress response than its role as a true biomarker (Wang et al. 2009). Many proteins identified in BALF are plasma proteins, as in our study complement C3,  $\alpha$ -1-antitrypsin, apolipoprotein A-1, and haptoglobin, and their increase in BALF is likely to be caused by increased permeability of the lung epithelium during CIPF and CB. However, these proteins are also produced locally in lung tissue to provide extra protection to the lung during disease processes (Wattiez and Falmagne 2005). Increased levels of complement proteins (Magi et al. 2002),  $\alpha$ -1-antitrypsin (Noël-Georis et al. 2001), and haptoglobin (Magi et al. 2002) as well as downregulation of apolipoprotein A-1 (Kim et al. 2010) have been detected in BALF from humans with IPF. Although no specific biomarkers for CIPF were found, the proteins with upregulated expression indicated activation of anti-inflammatory and glycolytic pathways, involvement of the complement cascade, and presence of cytoskeletal proteins in lung tissue of dogs with CIPF and CB.

## **6.4 TGF- $\beta$ -RELATED MOLECULAR PATHWAYS**

The central role of TGF- $\beta$ -mediated fibrosis is well established in human IPF (Koli et al. 2008), and activation of TGF- $\beta$  pathways has been suggested to take part in the pathophysiology of CIPF (Krafft et al. 2011a, 2012). TGF- $\beta$

has a wide variety of biological functions that contribute to development of fibrosis, such as recruitment of inflammatory cells, regulation of synthesis of ECM components, and induction of EMT in epithelial cells (Khalil et al. 1991, Rosendahl et al. 2001, Willis and Borok 2007). As CIPF shares clinical and histopathological features with human IPF/UIP and NSIP (Heikkilä et al. 2011a, Syrjä et al. 2013), we wanted to compare also the TGF- $\beta$ -related molecular pathways between canine and human diseases.

We demonstrated that enhanced TGF- $\beta$  signaling, indicated by increased P-Smad2 immunoreactivity in lungs of WHWTs with CIPF, is part of the molecular pathophysiology of CIPF and that the majority of TGF- $\beta$ -responsive cells were located in the altered alveolar epithelium, as seen in both UIP and NSIP (Study III). The finding of increased TGF- $\beta$ 1 signaling in CIPF lungs was confirmed in the study by Krafft et al. (in press 2014). The alveolar localization of active TGF- $\beta$  signaling with earlier reported marked atypia of AECII in CIPF (Syrjä et al. 2013) further supports the importance of abnormally activated alveolar epithelium in the pathogenesis of CIPF. The PaO<sub>2</sub> in WHWTs with CIPF seemed to correlate negatively with P-Smad2 immunoreactivity, possibly indicating that the fibrotic disease progression is related to more active TGF- $\beta$  signaling.

The ECM is not just a storage site for TGF- $\beta$ , but a place in which the structural components of ECM, most notably LTBP1s and fibrillins, participate in the regulation of TGF- $\beta$  availability and activation (Massam-Wu et al. 2010, Horiguchi et al. 2012, Todorovik and Rifkin 2012). The expression of ECM proteins LTBP-1 and fibrillin-2 has been shown to be upregulated in human IPF lung tissue (Leppäranta et al. 2012), but their role in CIPF or human NSIP had not been previously studied. In healthy human lungs, LTBP-1 and fibrillin-2 are only present around blood vessels and in arterial and bronchial smooth muscle cell layers (Leppäranta et al. 2012). We showed similar staining localization of LTBP-1 and fibrillin-2 in the lungs of control WHWTs (Study III). An interesting difference was seen in LTBP-1 staining intensity of AECs between UIP and NSIP. In NSIP, very mild or absent AEC staining was noted relative to the intense AEC staining seen in UIP samples, suggesting a different role of altered epithelium in these two human interstitial lung diseases. The alveolar LTBP-1 immunolabeling in CIPF WHWTs resembled that seen in UIP samples. As there was a significantly higher peribronchovascular LTBP-1 immunoreactivity in the lungs of CIPF WHWTs than in those of control WHWTs, the small airway component might be more important in CIPF. Previously, the presence of bronchiolar-derived epithelium on the peribronchiolar alveolar septa was detected by immunohistochemistry in the histological study of CIPF in WHWTs (Syrjä et al. 2013), indicating that the pathogenesis of CIPF involves epithelial differentiation or migration at the bronchio-alveolar junction. Although AECs play the key role in the fibrotic process seen in human IPF, it has also been shown that small airways are structurally altered and may

contribute to the remodeling events in both IPF/UIP and NSIP (Figueira de Mello et al. 2010).

The aberrant activation of developmental pathways has been suggested to be feature of the molecular pathobiology of human IPF (Sellman et al. 2008), and re-appearance of fibrillin-2, a developmental gene, has been previously detected in UIP lung tissue (Leppäranta et al. 2012). We detected alveolar interstitial immunoreactivity of fibrillin-2 in all diseased groups and especially in the fibroblastic foci in UIP lungs, as reported earlier (Leppäranta et al. 2012). Our finding may indicate that developmental pathways are activated also in CIPF and NSIP. Microarray analysis of CIPF lung tissue has previously shown upregulation of genes linked to developmental pathways (Krafft et al. 2013). However, as alveolar interstitial immunostaining of fibrillin-2 seen in CIPF lungs was generally less intense than that seen in human samples, fibrillin-2 expression might be a less prominent feature of CIPF.

Activins are cytokines belonging to the TGF- $\beta$  superfamily, and they are speculated to be the key intermediary in TGF- $\beta$ 1-mediated fibrosis (Patella et al. 2006, Hedger and de Kretser 2013). Strong evidence supports the importance of activins and their endogenous inhibitor, follistatin, in inflammation and fibrotic response, although studies regarding the role of activin B are sparse (Hedger et al. 2011, Hedger and de Kretser 2013, de Kretser et al. 2013). No studies on the role of activins in any canine respiratory disease have been published. We aimed to investigate the presence of activin A and activin B in normal canine lung tissue and to determine whether activins have a role in CIPF, in its AEs, and in canine ARDS (Study IV). The ARDS group was incorporated into the study since ARDS and AEs of CIPF are both histopathologically characterized by DAD (deClue and Cohn 2007, Study I) and since activin A has been suggested to take part in human IPF (Matsuse et al. 1996, de Kretser et al. 2012) and ARDS (Apostolou et al. 2012).

We demonstrated that activin B is strongly expressed in the pathologically altered alveolar epithelium in lungs of WHWTs with CIPF as well as in ARDS lungs. This novel finding supports the importance of activin B in fibrosis and in the pathological alteration of AEC. As activins share the same intracellular Smad signaling pathway with TGF- $\beta$ s (Itoh et al. 2000, Rosendahl et al. 2001), some of the increased Smad signaling seen in CIPF lung tissue (Study III) could be due to activin signaling. Based on the observed strong bronchial activin B immunoreactivity seen in control lung samples, the cellular origin of the activin B in the canine lung tissue under normal physiological conditions seems to be mainly the bronchial epithelium. Activin A, in contrast, seemed not to be a major protein expressed by canine lung tissue in either the normal or diseased state. This result contradicts findings in human IPF and ARDS (Matsuse et al. 1996, Apostolou et al. 2012) and in experimental bleomycin-induced mouse models (Matsuse et al. 1995) in which activin A overexpression has been demonstrated. An explanation for



this discrepancy could be that since there are significant species-specific differences in the baseline tissue expression of the  $\beta$ A and  $\beta$ B subunits (Hedger and de Kretser 2013) the expression of the  $\beta$ A subunit in canine lung tissue might be low overall.

Since AEs of CIPF often lead to euthanasia (Study I) and AEs of human IPF have very poor outcome despite treatment efforts (Collard et al. 2007, Meltzer and Noble 2008, van den Blink et al. 2010), predicting who is at risk of developing AE would be highly beneficial to guide early therapeutic approaches. Some biomarkers for human IPF AEs have been suggested, such as high serum levels of alpha-defensin (Mukae et al. 2002), SL2 protein (Tajima et al. 2003), and KL-6 (Yokoama et al. 1998), but none is in clinical use. A recent publication demonstrated that increased serum concentrations of activins A and B predicted higher mortality in critical care patients with acute respiratory failure (de Kretser et al. 2013).

Our findings of intense activin B and mild to moderate activin A immunoreactivity of alveolar edema in CIPF/AE and ARDS lung samples (Study IV) suggest that activins as soluble proteins might also be present in the serum of dogs suffering from acute respiratory failure and that especially activin B could serve as a potential biomarker of lung injury. The potential of activin B to serve as a marker of alveolar epithelial cell damage is further supported by our finding of activin B in the BALF of both of the CIPF WHWTs suffering from AE. No detectable levels were seen in control BALF samples from healthy WHWTs. Interestingly, two of the four BALF samples from WHWTs with CIPF but no AE showed very weak bands for activin B relative to the AE samples and two were negative. Further studies with alternative methods, such as ELISA, and with larger sample sets are needed to confirm and quantify the levels of activin B in BALF and sera of dogs with CIPF to better assess its use as an indicator of ongoing or higher risk of alveolar epithelial injury.

Since TGF- $\beta$  itself is not an ideal candidate for antifibrotic therapies, especially in chronic conditions, based on such harmful effects as profound immune dysregulation, seen in mice lacking TGF- $\beta$ 1, other therapeutic strategies targeting the TGF- $\beta$  system are of great interest (Böttinger et al. 1997, Leask and Abraham 2004). The potential of the activin-follistatin system as a therapeutic target in treating serious illnesses, e.g. different fibrotic diseases and ARDS, remains unknown, but blocking the actions of activins by an endogenous inhibitor, such as follistatin, might prove to be beneficial (Patella et al. 2006, Hedger and de Kretser 2013).

## **6.5 STUDY LIMITATIONS**

The main limitation in the follow-up study (Study I) was the low number of animals, resulting in a lack of significance in prognostic factor analysis for CIPF. The low number of animals is mainly due to CIPF being an uncommon

disease of mostly one breed and the diagnosis, as well as the verification of health, requiring invasive procedures. In addition, the median age of the healthy control WHWT group was slightly younger, and only three deaths occurred in the control group. However, the median follow-up time was similar in both groups, and the age was taken into account in the survival analysis. Overall, we consider our thoroughly examined healthy WHWT group and the histopathologically or HRCT-confirmed CIPF WHWT group unique for the descriptive study of the natural history of CIPF in WHWTs.

For the biomarker search from BALF (Study II), the number of samples from each group; CIPF WHWT, CB dogs, and controls, was small. However, the 2D-DIGE technique incorporates a pooled internal standard that minimizes the experimental variation associated with gel-to-gel comparisons (Lilley and Friedman 2004). Therefore, the changes in protein expression between the groups more likely reflect true biological differences. In addition, we chose not to include WHWTs in the CB or healthy control group to avoid possible subclinical CIPF carriers, and to further enhance characterization of CIPF and CB, we analyzed only protein spots detected in all of the samples in a particular group. The CB and control dogs were all adults and as the differences in protein profiles between CIPF and CB dogs were similar, breed and age were not likely to affect results.

For both immunohistochemistry studies (Studies III and IV), the main limitation was the low number of control lung samples, reflecting the difficulty in accessing normal lung tissue from old WHWTs. However, we chose not to include lung samples from other breeds, as CIPF is mainly breed predisposed. In addition, for Study IV, only two BALF samples from CIPF WHWTs suffering from AE were available for activin B analysis. This is mostly due to ethical reasons, as antemortem BALF samples are usually not taken from dogs with severe respiratory distress. The results of Western blot analysis of activin B in BALF of WHWTs with CIPF are therefore only indicative and require confirmation in larger sample sets.

## **6.6 FUTURE RESEARCH PROSPECTS**

IPF is a devastating disease of unknown etiology and pathogenesis in both humans and dogs. No curative treatment exists (Coward et al. 2010, Heikkilä et al. 2011a, Raghu et al. 2011). For human IPF, it has been shown that mortality rates from IPF have increased and are expected to continue to rise in the future (Olson et al. 2007). In addition, as a spontaneous animal model for human IPF is lacking (Gauldie and Kolb 2008), research on naturally occurring fibrosing lung disease in animals, such as CIPF, can shed light also on human research. Elucidating the molecular pathophysiology of human and canine IPF is crucial for better understanding of the disease mechanisms and for developing new treatment strategies and diagnostic markers. Because CIPF is mainly breed predisposed, genetic studies are needed to reveal the

complex genetic factors involved and later possibly to help dog breeders select healthier dogs for breeding purposes. Genetic studies on CIPF in WHWTs could also yield valuable information to research on the familial form of human IPF. In addition, studies on lung fibrosis in other dog breeds should be conducted to determine whether multiple fibrosing interstitial lung disease subtypes exist in dogs. Our findings regarding the natural history and molecular pathways of CIPF and the similarities with human IPF provide a good base for the planning of future research.

## 7 CONCLUSIONS

1. CIPF has a significant negative impact on life expectancy of diseased WHWTs. The median CIPF-specific survival of WHWTs after the onset of clinical signs was 2.7 years, but individual survival varied considerably, suggesting that CIPF in WHWTs may have either a rapid or a slow disease progression. No prognostic factors were identified. The 6MWD proved to be an easy and noninvasive parameter to evaluate lung function and level of exercise intolerance in WHWTs with CIPF.
2. No potential biomarkers specific for CIPF were found in proteomic analysis of BALF. However, six proteins were detected as differentially expressed in CIPF WHWTs and CB dogs relative to healthy control dogs, suggesting a common cellular response to disease processes in these two otherwise different lung diseases.
3. Increased TGF- $\beta$  signaling and expression of its ECM regulatory proteins LTBP-1 and fibrillin-2 were shown to be components of the molecular pathophysiology of CIPF, as also seen in human IPF/UIP and NSIP. The novel finding of upregulation of activin B in CIPF lung tissue and in diffuse alveolar damage suggests that activin B is part of the pathophysiology of CIPF and might act as a potential marker of alveolar epithelial damage.

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